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COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY

VOLUME XIII

COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY

Founded in 1933

by

REGINALD G. HARRIS

Director of the Biological Laboratory

1924 to 1936

The Symposia were organized and managed by Dr. Harris until his death. Their continued usefulness is a tribute to the soundness of his vision.

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Biological Applications of Tracer Elements

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FOREWORD

In the past the topics chosen for these Symposia have been broad problems in the borderline areas of biology, physics, chemistry, and mathematics, so that each summer the scientists participating in them have represented a variety of approaches but been interested in the same general problem. This year's Symposium departed from the established pattern by centering on a method applicable to a great many problems rather than a single one. Again it brought together scientists representing the borderline areas of the natural sciences, and thus accomplished one of its primary objectives.

The method considered at this year's Symposium on the *Biological Applications of Tracer Elements* is not new. It has been known for almost two decades. But recent discoveries in the field of atomic energy have made generally available materials that were very scarce, or unobtainable, only a few years ago; and consequently research with tracer elements is developing very rapidly and is assuming a leading role in many fields of modern biological science. Although this research is still in a very early stage, the accomplishments have been so significant that I felt justified in having them discussed by our Symposium.

In several conferences on tracer elements held recently, the emphasis has been on production and on methods for use. This Symposium, on the other hand, placed primary emphasis on the results obtained in biological experimentation; and the questions of production of tracer compounds, and of methods applied in their use, were considered only when essential for the understanding of results. The aims of the Symposium were to give a picture of the early biological work with tracer elements, and to present a general survey of problems now being investigated by these means. It was felt that such a survey would be useful in the planning of future research, and that the material currently available was not too extensive to be considered by our Symposium.

A reviewer of one of the recent volumes of the *Cold Spring Harbor Symposia* questioned the advisability of arranging the published papers alphabetically according to authors. He expressed a preference for arrangement according to topics. This question of the order of papers in the published volumes has received careful consideration. It is realized that for a first reading of the volume a topical arrangement would be preferable. In this year's material, for example, it would be logical to have Hevesy's "Historical sketch of the biological application of tracer elements" appear first, as it did on the program. The volumes of our Symposia, however, after being scanned or read once, are thereafter used extensively for reference. For easy location of a paper in the volume, alphabetical arrangement according to authors is much more convenient than a topical arrangement would be, and in order to facilitate reference use of the Symposia publications, the alphabetical order has been adopted in recent volumes. On this year's program the papers were arranged, as usual, according to subject matter. Gemmill's paper on "Isotopes in pharmacodynamics" was not presented on the program, but is included in this volume.

The program for this Symposium was organized with the help of the directors of the biology divisions of the three National Laboratories of the Atomic Energy Commission—A. M. Brues (Argonne), A. Hollaender (Oak Ridge) and L. F. Nims (Brookhaven)—M. D. Kamen, A. Mirsky, and D. Rittenberg. The editorial work was done by Dr. Katherine Brehme Warren.

The Symposium was held from June 8 to June 16, 1948. The registered attendance was 163. During the meetings one half-day was spent in visiting the Brookhaven National Laboratory. The expenses of our foreign guests were covered by a grant received from the Carnegie Corporation.

M. DEMEREC

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CHROMOSOME BREAKAGE INDUCED BY ABSORBED RADIOACTIVE PHOSPHORUS

T. J. ARNASON

Any process which speeds the mutation rate is or can become of biological importance, since mutations provide the raw materials of evolution. The diversity found in present-day organisms has been made possible by the occurrence of mutations in the past. The evolution directed by plant and animal breeders similarly depends on genetic variability present in the breeding stock. If direction along particular lines should be hampered by lack of suitable variants, then waiting for the desired mutation to occur is likely to become tedious since the spontaneous mutation rate, although variable for different genes, is usually very low, ranging between one in several thousand to one in over a million gametes. It appears to be possible, therefore, that methods developed for raising the mutation rate may become of practical as well as of theoretical value.

Although gene mutation is basic to the process of evolution, another way in which hereditary variations may be produced is by chromosome breakage followed by loss of fragments or rearrangement of the pieces. If the structural changes alter the numerical relations between genes, *e.g.*, when there is duplication or deficiency, notable changes in the development of the organism having the altered genotype often follow. Even if there is no gain or loss rearrangement may result in modified effects of certain genes, the so-called "position effect." Finally, since individuals heterozygous for structural rearrangements often have reduced fertility, the homozygous segregates tend to become to a greater or lesser degree isolated from the remainder of the population.

From several pieces of earlier work, it appears that the number of gene mutations and chromosome breaks produced by ionizing radiations in living tissue is directly proportional to the dosage, *e.g.*, to the number of roentgens of X-rays (Lea, 1946). The effectiveness of X-rays is particularly well established but considerable information has been obtained also regarding mutations induced by alpha, beta, gamma and neutron rays. Until very recently, the radiation source has always been external to the irradiated cells. It is now possible to transfer a source of effective radiations into living cells and even into the genetically active parts of cells, the chromosomes, with their contained genes. It has become possible because the radioactive isotopes of elements usually occurring in chromosomes and other protoplasmic structures or ingredients are now available for biological work.

With the increasing availability of radioactive isotopes and the increasing possibility or even probability of their widespread dissemination, it becomes of considerable interest to establish the effects of absorbed radioisotopes and to compare the effects produced with those resulting from other types of radiation. It is possible that certain radioisotopes may be especially effective for mutation production because of their concentration in chromosomes; they are then within the genetic target area and can hardly miss when energetic particles are emitted. It is possible also that, when recoil of an atom nucleus occurs within a chromosome, genetically significant molecular rearrangements may ensue.

If it is established that absorbed radioisotopes are effective in inducing mutations, they offer the following advantages for some types of work:

(1) Low dosage per unit of time may be administered, conveniently, for a lengthy period.
(2) Large numbers of plants and possibly of some animals, may be treated more conveniently during some stages of development, *e.g.*, the period of rapid growth and maturation, by this method than by X-rays.
(3) Additional fundamental information relating to the mutation process may be gained from controlled experiments designed to determine the mutagenic effects of different absorbed radioisotopes.

Some rather obvious disadvantages connected with the use of radioactive materials for mutation induction are: (1) The exact dosage is difficult to determine and control; also, it cannot be stopped at will; (2) unceasing vigilance must be exercised to prevent contamination of personnel, instruments and the surroundings generally.

Not all radioisotopes are equally suitable for raising the mutation rate. Factors to consider in the choice of a radioisotope for mutation induction include: (1) The rate of decay; the half-life of different isotopes varies from something less than four seconds to many years (Kamen, 1947); (2) the type of rays emitted (alpha, beta, or gamma); (3) the hardness of the rays; (4) the freedom of movement of the active element within the organism; phosphorus moves freely within plants (Kamen, 1947), whereas strontium absorbed by young plants is disposed of by the plant in the first few leaves; (5) some elements may be toxic even at high dilution, *e.g.*, boron or mercury; (6) finally, if the absorbed element is one which tends to be concentrated in meristems (plants) or gonads (animals), and particularly in chromosomes, it ob-

viously has a position advantage over elements which do not.

Besides the use of absorbed radioisotopes, certain other methods of obtaining radiations originating within cells are feasible. One is by inducing uptake of boron or lithium, then bombarding with slow neutrons to give rise to large fission fragments and columnar ionization. Allowance must of course be made for the direct neutron effects. By such methods the dosage could be controlled, and, as with radiations from an external source, the time period for irradiation would normally be short.

Our investigations, organized on a co-operative basis, involved Dr. J. W. T. Spinks of the Chemistry Department, two student assistants, Elaine Cumming and R. L. Irwin, and myself. Our immediate objectives were (1) to determine the amount of P^{32} that could be tolerated by germinating seeds and young seedlings, and (2) to determine the effects on chromosomes of absorbed sub-lethal amounts (Arnason, Cumming and Spinks, 1948a and b).

Table 1. The Number of Different Aberrations Found in Microsporocytes of P^{32}—Treated Plants

Plant	Number of plants	rd. of P^{32} per plant at start	Number of cells counted	Number of aberrant cells[1]	Number of different re-arrangements
Barley	16	0	469	0	0
	24	.00065	72	0	0
	24	.0065	115	0	0
Einkorn wheat	18	0	332	0	0
	24	.00065	170	26	1
	24	.0065	112	0	0
durum wheat	19	0	422	0	0
	24	.00065	215	23	3
	24	.0065	172	30	4
vulgare wheat	24	0	1122	0	0
	24	.00065	160	9	2
	24	.0065	230	24	6
	13[2]	.35	522	119	3

[1] Rearrangements recorded only if they occurred in two or more microsporocytes.
[2] Plants germinated and grown in soil to which .35 rd. of P^{32} was added for each plant. Much less than this amount was absorbed.

Materials and Methods

Radiophosphorus was chosen for several reasons. (1) It was one of the few radioactive isotopes available to us. (2) Phosphorus is readily absorbed, moves freely within plants and becomes somewhat concentrated in meristematic or rapidly growing parts. (3) The half-life (14.3 days) is considered favorable. (4) The emitted radiation consists of beta rays, relatively pure. (5) Phosphorus is well represented in nucleic acids which in turn are abundant in chromosomes.

All counts and measurements of radioactivity were done or supervised by Dr. Spinks. His description of the method of counting follows: "The activities of solutions were determined by evaporating down an aliquot on a platinum dish which was then placed under the window of an end-on Geiger Muller chamber, having a thin window of mica, and counted. The chamber was connected to a scale of 128 scaling circuit and any sample was counted for a sufficient length of time to give approximately 10,000 counts (standard deviation is then only about 1 percent). By choosing a suitable aliquot, the rate of counting was kept below 2000 counts per minute and the correction for the resolving times of the tube (2×10^{-4} seconds) was then less than one percent.

"Plant materials were wet ashed before counting and, where necessary, corrections were made for self absorption by the active material. The natural rate of the counting tube (background) was allowed for by subtracting it from the total count for any sample. To allow for decay an aliquot of the original stock solution used in an experiment was evaporated to dryness and counted immediately before and after counting the unknown material. To calculate the absolute number of disintegrations, the geometry factor was determined. In these experiments, the geometry factor was 3.9."

Species used in our preliminary investigation included *Triticum vulgare* Vill. (n = 21), *T. durum* Desf. (n = 14), *T. monococcum* L. (n = 7) and *Hordeum distichon* L. (n = 7). Seeds of each of these species were germinated in individual test tubes containing .1 ml. nutrient solution and measured amounts of P^{32} to determine how much activity could be tolerated under those conditions. In the small tested group, germination was completely arrested or inhibited in all seeds if the P^{32} concentration was .65 rd. or over, .065 rd. proved

lethal to many, while a concentration of .0065 rd. had scarcely discernible effects on germination and growth (Spinks *et al.*, 1948).

To determine whether mutations may be induced by seedling-absorbed P^{32} within the tolerated range 24 seeds of each species were germinated in .0065 rd. solution of P^{32} and an equal number of seeds of each kind was germinated in solution containing .00065 rd. P^{32}. The initial amount of solution per test-tube was .1 ml. When the test-tubes became dry a small quantity of nutrient solution containing neither activity nor phosphorus was added. At the end of 13 days the seedlings were transplanted to one gallon crocks containing untreated soil and grown to maturity. Tests of residues in 10 test-tubes indicated that 90 percent (standard deviation 4 percent) of the P^{32} had been absorbed by the plants.

FIGS. 1-5. Chromosome aberrations in P^{32}-treated plants.
FIG. 1. Telophase bridge in a cell of a barley root-tip.
FIG. 2. Chromosome fragment in a *durum* root-tip cell.
FIGS. 3 and 4. Large lagging fragments in *durum* microsporocytes.
FIG. 5. Anaphase bridge in *vulgare* microsporocyte.

Cytological material collected from treated plants and controls included both root-tips and anthers. Fixation was in acetic-alcohol (1 : 3). Root-smears were stained by the Feulgen technique and pollen mother cell smears were stained with iron-aceto-carmine. Preparations were made permanent by mounting in diaphane.

Some additional material for cytological examination was obtained from plants grown in soil to which had been added .35 rd. of P^{32} in the form of $Na_2H\,PO_4$ for each plant.

RESULTS

Only six control plants of each species were grown with the seedling-treated group. Subsequently microsporocytes from additional untreated plants were examined and the results of these are included in Table 1. In none of these plants was any aberration found repeated in two or more cells of an anther. Two univalents were occasionally found at diakinesis or metaphase. Their presence may be attributed to premature separation of homologues or to failure of chiasma formation in earlier prophase stages. It is thought probable, therefore, that chromosomal irregularities are rare in the untreated populations from which the experimental plants were taken.

Chromosome fragments and anaphase bridges (Figs. 1 and 2) were observed in root-tip cells collected from treated plants within five days of the start of germination. The number seen was highest in the 42 chromosome wheat, lowest in 14 chromosome wheat, and barley. Bridges may be formed either by fusion of broken ends of sister chromatids or by translocation involving fusion of broken ends of two chromatids or chromosomes, both of which have centromeres.

Some of the main results of the cytological study of microsporocytes of treated plants are presented in Table 1. Aberrations are listed in the table only if two or more cells of an anther had configurations that could be attributed to the same rearrangement. In all groups of this kind the structural changes must have occurred in an earlier generation of cells and the new condition has proved its capacity to survive the mitotic cycle.

It is probable that most of the breaks and rearrangements occurred while the plants were young since at that time the P^{32} activity was highest and the concentration was also high because of the relatively small mass of the plants. Aberrations seen included: single univalent chromosomes, several univalents in each cell, chromosome fragments, chromosome bridges and fragments at anaphase and chains of three or more chromosomes (Figs. 3-5). The exact nature of the original change could often not be stated with the precision obtained by Giles (1947) and others working with treated Tradescantia microspores. Any process which interferes with chromosome division or movement may result in elimination of that chromosome, *e.g.*, inactivated centromere or breakage followed by certain types of rearrangement. Most first division bridges with

fragments probably result from inversions (McClintock, 1941). Some second division fragments were seen; these are doubtless derived from first division fragments that have been included in daughter nuclei.

In one *vulgare* plant grown on treated soil, the pollen mother cells had a large number—up to 10—of univalents indicating that many chromosomes had been completely eliminated or that homologies had been extensively altered. It seems most probable that high local concentration of P^{32} was responsible.

In some anthers all cells appeared normal with one or two exceptions. Aberrations appearing thus, singly, may be taken as an indication that breakages and rearrangements are still occurring in microsporocytes, although by this time (6 weeks) the P^{32} has decayed to about one eighth of its original activity and the absorbed active element has been diluted in the increasing volume of the growing plant. In spite of the loss of activity and the increase in plant volume, prolonged mutagenic effectiveness of seedling-absorbed P^{32} may occur if the active element is not evenly dispersed through the growing plant but reaches high local concentration in cells of the germ track. Measurement of the amount of activity in anthers of a single plant grown in treated soil, indicated that 5.7 percent of the P^{32} present in the harvested plant (roots not included) was in the anthers. The mass of the anthers was calculated to be only 0.28 percent of the plant mass. The slightly younger anthers of a second plant formed .05 percent of the mass of the plant including roots.

The frequency of chromosome breaks induced by absorbed P^{32} must be determined by other experiments. It appears likely that, as with X-rays, breakage is proportional to dosage. Since, however, the dosage rate per unit of time is low, the proportions of rearrangements involving two breaks may also be low as compared to those produced by corresponding total dosage of X-rays. It is possible, however, that the ionizing particles from the P^{32} which decays within chromosomes produce more than one break with a high frequency compared to X-rays.

Observed aberrations were most frequent in the hexaploid and tetraploid wheats. Only one aberration involving many cells was found in a diploid species. Since anaphase bridges were also more common in root-tips of young treated polyploid seedlings than in the diploids it may be supposed that increase in chromosome number and length increases the chances of breakage and rearrangement. Unbalanced changes doubtless have, in addition, a better chance of survival in polyploids than in diploids since gene loss in a diploid may interfere with vital functions while corresponding losses in a polyploid may have little effect because of protective duplicate genes still present in other chromosomes.

Indirect evidence of the occurrence of some drastic mutations comes from observations made on seeds from treated plants. In 4 out of 74 harvested *vulgare* heads all the seeds were split, flattened and shrivelled. In two additional heads some of the seeds were shrivelled, others plump. In treated *durum* and barley also there was apparent endosperm failure in some seeds. No similar blasted seeds were found in untreated plants grown at the same time nor in any grown subsequently. Cytological study of the progeny of treated plants is just beginning but already Mrs. Cumming reports that some of the plants are probably heterozygous for rearrangements. Rings of four chromosomes found in the microsporocytes of one *vulgare* plant indicate that a reciprocal translocation has been transmitted by the treated parental plant.

Whether gene mutations have been induced in the treated individuals has not been determined. Species of wheat, particularly those that are polyploid, are not favorable material for the study of gene mutation rates (Stadler, 1931).

Discussion

The primary mechanism responsible for the observed meiotic aberrations is doubtless chromatid or chromosome breakage induced by ionizing beta particles from P^{32}. Except in the group grown in treated soil, the radiation dosage was small—ten roentgen-equivalents-physical, or less per gm. of tissue per day at the start of the experiments (Spinks *et al.*, 1948). Carlson (1941) has stated that there is no minimum dosage of X-rays below which chromosome breakage will not occur. He stated also that in grasshopper neuroblasts treated with 125 r an average of one to two fragments per cell was produced. Our investigation does not give information on the primary rate of chromosome breakage; it merely indicates that some breakage has occurred following absorption of the small measured amounts of P^{32} which were used. Since phosphorus is well represented in nucleic acids and nucleic acids are particularly abundant in chromosomes it seems likely that a flow of phosphorus to rapidly growing structures will be maintained. If there is concentration of P^{32} in meristems and in developing floral organs, particularly sporangia, production of mutations that may reach germ cells will be favored. Radioautographs of 12-day and 35-day old wheat seedlings showed fairly general distribution of seedling-absorbed P^{32}. On the other hand activity counts showed that there was considerable concentration of P^{32} in the growing anthers of a corn plant five days after injection with P^{32} solution in one of the lower stem internodes.

The mutagenic effectiveness of P^{32} may be enhanced by its inclusion in the nucleic acids and the nucleoproteins of chromosomes. Beta particles emitted from within the chromosome must be especially likely to cause chromosome breakage or other genetic changes since a "hit" on the chro-

mosome may be regarded as certain and multiple "hits" are possible.

The recoiling atom nucleus is almost certain to have enough energy to break any chemical bond (Libby, 1947). According to Lea (1946), 4 e v of energy is sufficient to release the C-H bond in a molecule, and the energy of the recoil of a P^{32} nucleus will usually exceed this. In addition, P^{32} with an atomic number of 15, becomes converted to S^{32}, atomic number 16, with a change in valence from 5 to 2. Thus at the point of decay there must be some molecular rearrangement; if the rearrangement involves a gene molecule the expected result is a mutated gene.

It appears probable that absorbed radioisotopes will cause genetic changes as readily in the germ cells of animals, including man, as in the sporocytes of plants. For this reason radioactive elements should be used with caution in the treatment of disease in humans. Since mutations are usually harmful, even small increases in the mutation rate should be avoided if possible. Doubtless some radioisotopes are more effective than others in raising the mutation rate. The active isotopes of elements which are included in chromosomes probably will prove to be among those which can induce germinal mutations with a relatively high frequency.

Acknowledgments

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THE PATH OF CARBON IN PHOTOSYNTHESIS[1]. III

A. A. BENSON AND M. CALVIN

Although the overall reaction of photosynthesis can be specified with some degree of certainty ($CO_2 + H_2O$ + light → sugars + possibly other reduced substances), the intermediates through which the carbon passes during the course of this reduction have, until now, been largely a matter of conjecture. The availability of isotopic carbon, that is, a method of labeling the carbon dioxide, provides the possibility of some very direct experiments designed to recognize these intermediates and, perhaps, help to understand the complex sequence and interplay of reactions which must constitute the photochemical process itself.

The general design of such experiments is an obvious one, namely the exposure of the green plant to radioactive carbon dioxide and light under a variety of conditions and for continually decreasing lengths of time, followed by the identification of the compounds into which the radioactive carbon is incorporated under each condition and time period. From such data it is clear that in principle, at least, it should be possible to establish the sequence of compounds in time through which the carbon passes on its path from carbon dioxide to the final products. In the course of shortening the photosynthetic times, one ultimately arrives at the condition of exposing the plants to the radioactive carbon dioxide with a zero illumination time, that is, in the dark. Actually, in our work the systematic order of events was reversed, and we have begun by studying first the dark fixation and then the shorter photosynthetic times.

The results of the beginnings of this sort of systematic investigation are given in Table I which includes three sets of experiments, namely a dark fixation experiment and two photosynthetic experiments, one of 30 seconds duration and the other of 60 seconds duration.

The method that was developed for separating the various components of the cell is shown in diagrammatic form in Figure 1. Six fractions were obtained, and on the basis of the method of fractionation, certain general properties of the compounds contained in each of these fractions can be specified.

The insoluble fraction, which contained practically none of the very quickly formed radioactive products, consists of the high molecular weight substances such as the proteins, cellulose and starch together with the very water-insoluble low molecular weight materials such as the fats and pigments. Fraction I will contain those materials which can be ether extracted from an acid aqueous medium by a continuous operation extending over a period of fifteen hours. These consist of the fatty acids and the di- and tricarboxylic acids as well as the lower hydroxylated carboxylic acids such as malic acid, lactic acid, glyceric acid and citric acid. The higher polyhydroxy acids such as gluconic acid and the phosphate esters and anhydrides would not be extracted under these conditions. Fraction II will contain those substances which are or can be cationic. This is limited to the nitrogenous bases, amino acids and oxonium compounds. Fraction III consists of those anionic materials which could not be ether extracted, namely the organic phosphates and highly hydroxylated carboxylic and enolic acids. Fraction IV consists of the non-ether extractable neutral molecules, that is, the simpler carbohydrates.

Fraction III was further separated into two parts. Part A consists of that group of anionic

TABLE 1. CO_2 FIXATION BY SCENEDESMUS

	10 min. prcillumination 1 min. dark fixation	30 second photosynthesis	60 second photosynthesis
Total Fixed/cc. cells (c.p.m. 10^{-6})	0.97, 100%	6.2, 100%	12, 100%
Insoluble	0%	0%	5%
I. Ether extract at pH 1	12%	10%	3.7%
II. Amino acids	39%	11%	3.7%
III. A. Sugar phosphates	4.2%	44% (2% III A, 97% III B, 0.5% IV)	59% (29% III A, 64% III B, 15% IV)
III. B. Phosphoglycerate	42%	27%	20%
IV. Sugars	0.1%	4.7%	4.1%

[1] This paper is based on work performed under Contract #W-7405-Eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley, California.

substances elutable off an anionic exchange resin by ammonia. Part B consists of those anionic materials which are not elutable with ammonia but which can be removed from the resin with sodium hydroxide. So far, the only substance which we have found to display the behavior of Fraction III-B on the anion resin is phosphoglyceric acid.

A relatively limited group of compounds are known to exhibit the behavior corresponding to which is apparently unchanged by contact with ammonia and evaporation in all probability is the more difficultly hydrolyzable hexose phosphate such as glucose-6-phosphate, although it might also contain substances such as gluconic or mucic acids.

The specific identification of most of the radioactive components in these fractions has already been described (Calvin and Benson, 1948; Calvin *et al.*, in press). A more detailed identification of

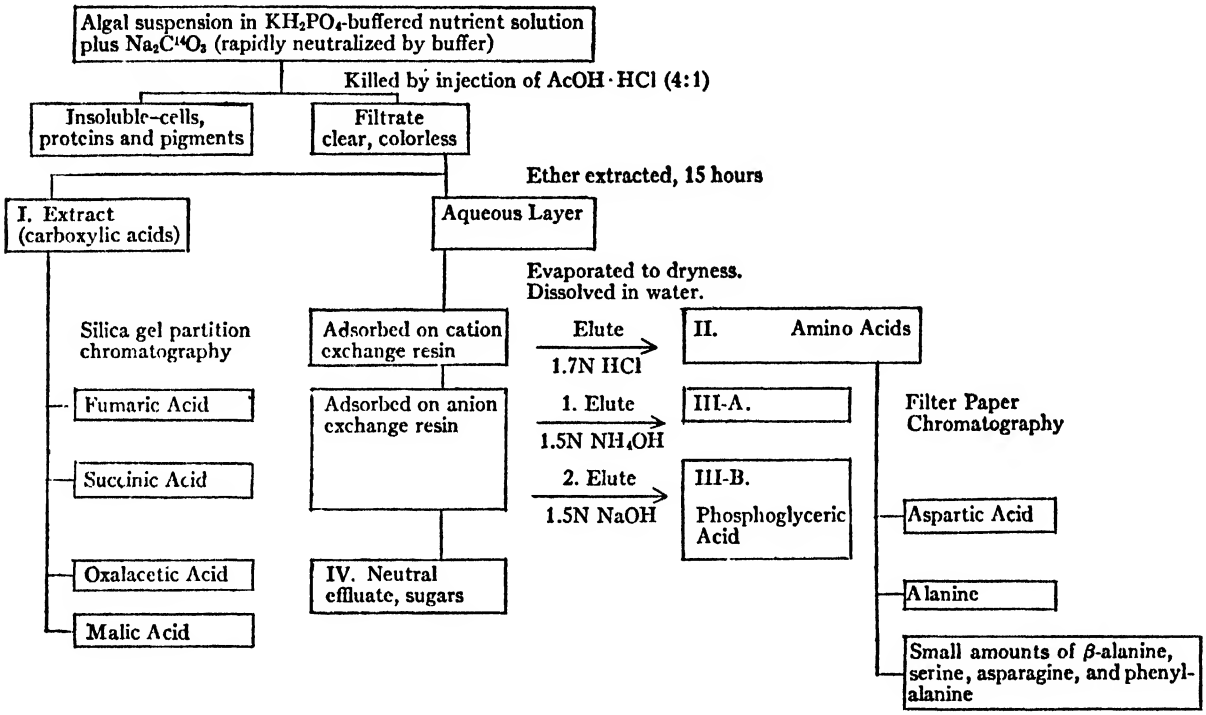


FIG. 1. FRACTIONATION OF RADIOACTIVE PRODUCTS OF PHOTOSYNTHESIS BY ALGAE

Fraction III-A. These consist of the sugar phosphates, both hexose and triose, together with substances like gluconic and mucic acids. An additional characteristic of Fraction III-A makes possible its further breakdown into three more groups of radioactive components.

If the original Fraction III-A ammonia eluate is concentrated by vacuum evaporation and then passed again through the anion column, one finds that all of it is no longer absorbable on the resin, and, furthermore, of that which is absorbed, a certain fraction has become non-ammonia elutable and has been identified with the original III-B fraction, namely phosphoglyceric acid. That part of Fraction III-A which is very readily converted to phosphoglyceric acid we believe to be triose phosphate (Calvin and Benson, 1948). That part of III-A which becomes non-absorbable we believe to be easily hydrolyzable phosphate esters such as glucose-1-phosphate, while the remainder of Fraction III-A the amino acid fraction by means of radio autographs of paper chromatograms has also been described (Stepka, Calvin and Benson, in press). Therein, the presence of radioactive aspartic acid, alanine, asparagine, β-alanine, serine, and phenylalanine was demonstrated. Although large quantities of glutamic acid were always present, it was never found to be radioactive.

An examination of the three experiments given in Table I reveals smooth trends between all of the three conditions in each of the fractions and in the general nature of the distribution. There is no sharp discontinuity between the photosynthetic experiments and the dark fixation experiment. This indicates a close relationship between the dark fixation and photosynthesis. However, some doubt existed as to the significance of this dark fixation in photosynthesis. This arose from the demonstrated reversal of certain decarboxylation reactions found in non-photosynthetic organisms. At the present

writing, these consist of only the two following reactions:

1. CO_2 + pyruvic acid ⇄ oxalacetic acid (Evans *et al.*, 1943).

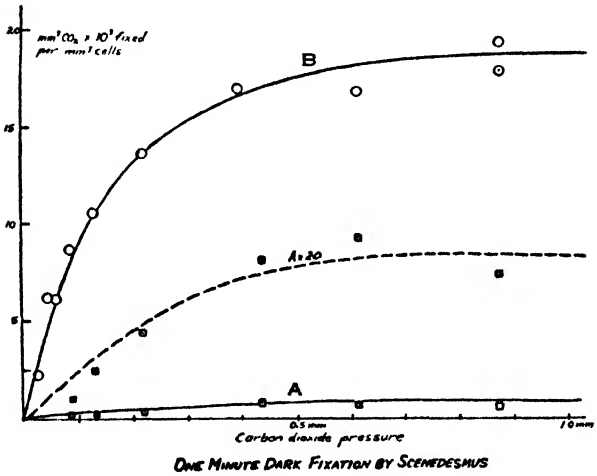
2. CO_2 + ketoglutaric acid ⇄ oxalsuccinic acid (Ochoa, 1948, p. 145).

In view of the absence of radioactivity in any of the components of the tricarboxylic acid cycle having more than four carbon atoms under any circumstances in the early products of photosynthesis, we need only consider Reaction 1. That the dark fixation following preillumination is not due to the simple reversibility of respiratory or fermentative reactions has already been demonstrated (Calvin and Benson, 1948) and is readily apparent from the mere fact of its great dependence upon preillumination of the cells in the absence of carbon dioxide. The suggestion purporting to account for the increased dark fixation following preillumination depends upon a net mass action reversal of the decarboxylation reaction and those leading up to it by the increased carbon dioxide concentration which obtains upon the addition of radioactive carbon dioxide. The way in which this might be brought about is illustrated by the following reaction schemes:

$$\begin{array}{l} \overset{h\nu}{\downarrow} \\ CO_2 \rightarrow A \rightarrow B \rightarrow C\text{---} \rightarrow \text{Products} \\ + \\ CH_3COCO_2^- \\ 1. \quad \uparrow\downarrow \\ {}^-O_2C\text{—}CH_2\text{--}CO\text{—}CO_2^- + R \rightleftarrows {}^-O_2C\text{—}CH_2\text{—}CHOH\text{—}CO_2^- + Ox \end{array} \qquad 1a.$$

During the preillumination in the absence of carbon dioxide, the residual carbon dioxide concentration (of necessity intra-cellular) is reduced by the normal photosynthetic mechanism through a series of intermediates, A, B, C, etc., presumed unknown. This by hypothesis is the only effect of the preillumination in the absence of carbon dioxide. Upon the addition of the radioactive carbon dioxide, the carbon dioxide concentration is increased producing a mass action reversal of a sequence of reactions to and through the decarboxylation reaction. For very small amounts of carbon dioxide fixed with respect to the total fixing capacity, that is, the saturation value, the amount fixed should be simply proportional to the increase of carbon dioxide concentration and independent of the total carbon dioxide concentration.

Thus, if a plot is made of the amount fixed against the carbon dioxide added, the initial slope of this curve should be independent of the total amount of carbon dioxide present. Such a plot is shown in Figure 2. The initial slope for the preilluminated algae, curve B, is over one hundred times that for the non-preilluminated algae, curve A. The ordinates are calculated from the initial specific activity of the carbon fed for both curves. If the difference between the two curves is due to a higher initial non-radioactive carbon dioxide concentration in the predark cells, the added radioactive carbon dioxide would have been more dilute (lowered specific radioactivity) in these cells than in the preilluminated cells by the ratio of the initial non-radioactive carbon dioxide concentrations. In order to make the slope the same, the ratio of residual carbon dioxide concentrations in the predark cells to that in the preilluminated cells must have been greater than one hundred, that is, the preillumination must have reduced the residual carbon dioxide concentration in the cells by a factor of over one hundred.



One Minute Dark Fixation by Scenedesmus

FIG. 2.

Since the cells were being continually swept with carbon dioxide-free helium at a rate of about 500 cc. a minute throughout the entire experiment which lasted about an hour, of which the first half hour consisted merely of sweeping the cell suspension in the dark, the residual carbon dioxide concentration within the cells could not have been greater than that corresponding to a partial pressure of the order of 0.1 mm. Therefore, the preillumination would have had to reduce the carbon dioxide concentration to a value corresponding to a partial pressure of less than 0.001 mm. This we know the light cannot do.

The rate of photosynthesis in a wide variety of green plants begins to fall off in the vicinity of 1.0 mm. partial pressure of carbon dioxide. In a medium containing one-fifth atmosphere of oxygen, the steady state carbon dioxide partial pressure can-

not be reduced below approximately 0.1 mm. This is, of course, due to a balance between the photosynthetic rate of removal of carbon dioxide and the production of carbon dioxide by respiratory and fermentative mechanisms. While it is true that in our case (anaerobic), carbon dioxide is presumably produced only by a fermentative path, this would also produce a corresponding lower maximum possible residual carbon dioxide concentration in the predark algae and the lower limit for the steady state carbon dioxide partial pressure would be reduced in the same ratio. It is, perhaps, significant that the dependence of the dark fixation on carbon dioxide partial pressure shown in Figure 2 resembles very much the dependence of photosynthetic rates on carbon dioxide concentration.

The curves in Figure 2 can very readily be understood if the function of the preillumination is to increase the concentration of the reducing agent R in Reaction 1a) and of the carbon dioxide acceptor (s), such as the pyruvate, through some cylic path. Such a cycle has already been presented (Calvin and Benson, 1948; Benson *et al.*, in press), and is reproduced here although the various experimental data leading up to it will not be reviewed again in this paper. The two reactions labeled with a (?) are introduced as possible routes to account for the appearance of a considerable amount of radioactivity in the carboxyl groups of succinic acid and alanine when carboxyl-labeled acetate is fed to the algae.

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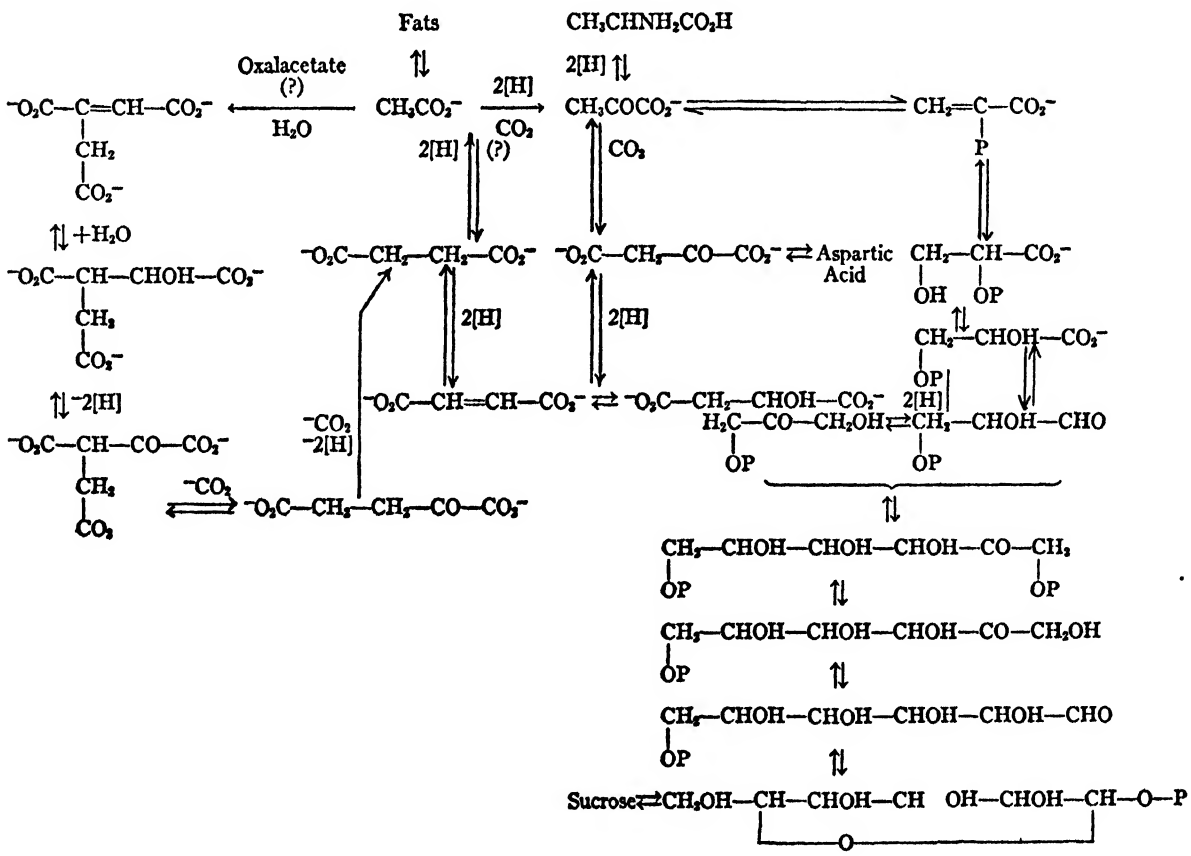
Discussion

Wood: It is indeed gratifying to learn of these results by Dr. Calvin. I have been interested in this problem for some time and while at the University of Minnesota, Dr. George Burr and I set up experiments to determine the distribution of fixed $C^{13}O_2$ in sugars isolated from Kentucky Wonder beans.



It was found, just as has been reported by Dr. Calvin, that the isotope distribution was highest in the 3,4 positions, next highest in 2,5 positions and lowest in the 1,6 positions. In these experiments the beans were allowed to photosynthesize about one-half hour in the presence of $C^{13}O_2$. Dr. Burr left Minnesota in 1946 to go to Hawaii, and I left for Western Reserve, so the experiments were discontinued.

Dr. Burr later sent some C^{14} sucrose to me from Hawaii which he had isolated from sugar cane. Here again a similar type of distribution of the isotope was found.

In the experiments with beans an interesting observation was made; the sucrose had a high concentration of isotope whereas a fermentable reducing sugar, presumed to be glucose, had a low isotope concentration. The results seem to indicate that the fixed carbon was transferred to sucrose more rapidly than to glucose. This problem appears to offer possibilities for further interesting studies.

The mechanism of fixation of CO_2 as proposed by Dr. Calvin utilizes two fixation reactions both of which are known to occur in biological systems. Utter, Lipmann and Werkman have shown with labeled acetate that acetate can be converted to pyruvate by *E. coli*. The reaction as studied was with formate but *E. coli* can reduce CO_2 to formate so the over-all reaction could be considered.

$$CO_2 + \text{acetate} \rightarrow \text{pyruvate}.$$

The fixation of CO_2 in oxalacetate is by now a generally accepted reaction, although it should be emphasized that even in this reaction the details are unknown. It is not known for example how ATP functions in this reaction.

The cleavage of succinate to two molecules of acetate has not as yet been satisfactorily established in a well defined system, although it has long been considered as a likely reaction.

It is by no means established, of course, that these reactions take place in photosynthesis but it is an attractive hypothesis. From the standpoint of comparative biochemistry it seems most probable that many of the same reactions should be utilized in photosynthesis as are utilized in fixation of CO_2 and in the general metabolism of bacteria and animals. As a working hypothesis it may be advantageous until evidence appears to the contrary to consider that the main difference between photosynthetic and non-photosynthetic utilization of CO_2 is the source of the energy for the reduction and that the light reaction is linked intimately with this reduction. At any rate the distribution of the isotope in the sugar is precisely that expected by the known fixation reactions as found in non-photosynthetic metabolism and it is probably more than a coincidence that this is the case.

It should not be implied that these findings take us very far toward an understanding of photosynthesis. They show what systems may be operating but the detailed work is yet to be done. It is necessary to prove these systems are operating, to find out the details of the mechanism of the reactions and most important to uncover the link between the light reaction and the fixation of CO_2.

LIPMANN: Dr. Calvin proposes as a step in photosynthesis the condensation of acetic acid and carbon dioxide. Such a reaction is rather well supported by enzyme chemical observation. Dr. Wood referred already to the appearance of marked carbon dioxide in the carboxyl group of lactic acid in experiments with *Clostridium butylicum*.

There seem good reasons to suspect that such an alpha-carboxylation would be the reversal of the phosphoroclastic split of pyruvate to acetyl phosphate, CO_2, and H_2 as observed in extracts of *Clostridium butylicum*. I would suggest, therefore, that in Dr. Calvin's scheme an energy-rich phosphate be added to prime the acetate for condensation with Co_2.

$$CH_3COOH + \sim ph + CO_2 + 2H \rightarrow CH_3COCOOH + ph + H_2O$$

This type of alpha-carboxylation had previously been considered tentatively as a step in photosynthesis by ourselves and by Dr. Ruben; and it is gratifying to see this possibility gaining ground with Dr. Calvin's recent observations.

THE USE OF THE O^{18} ISOTOPE

RONALD BENTLEY

The non metal elements concerned in biochemical reactions have all been extensively studied by isotopic methods, with the exception of oxygen. Although heavy isotopes of oxygen were discovered in 1929, isotopically enriched samples have not been readily accessible. A limited supply of water containing O^{18} is now available from the U. S. Atomic Energy Commission, and a number of laboratories are developing methods which will yield more highly concentrated material. With increased supplies of O^{18}, it will be possible to trace all the major elements of biochemical interest. This Symposium provides an opportunity to outline results which have already been obtained using O^{18}, to review some special problems connected with this isotope, and to describe current studies in which it is being utilized.

Normal oxygen is a mixture of three stable isotopes, which have the isotopic weights and normal abundances given in Table 1. The abundance ratio

TABLE 1. THE ISOTOPES OF OXYGEN

Mass Number	Isotopic Weight	% Abundance	Half-life	Radiation
15	15.0078		126 secs.	$\beta+$
16	16.00000	99.757		
17	17.00450	0.039		
18	18.00490	0.204		
19	19.0139		31 secs.	$\beta-$ and γ

varies somewhat with the source: an account of these variations and of their biological significance will be given later. Two radioactive isotopes, O^{15} and O^{19} have been produced, but on account of their short half-life periods it is unlikely that they will be used in tracer studies. The more abundant isotope, O^{18}, has been concentrated in a number of ways: such processes also concentrate the rarer O^{17} isotope, but the extent of this concentration is small and so for tracer work, O^{18} must be used.

Only a slight concentration of O^{18} takes place during the electrolysis of water and a better separation has been obtained by fractional distillation. Urey and his co-workers devised a column of alternate stationary and rotating cones, which produced water with a maximum O^{18} concentration of 0.85 percent (Huffman and Urey, 1937). From a cascade of three fractionating columns, water containing 1.3 percent of O^{18} was obtained (Thode, Smith and Walkling, 1944), and other columns have been described (Brodskiĭ and Skarre, 1939; Dostrovsky and Hughes, 1946). For the preparation of almost pure O^{18}, the method of thermal diffusion was used. The equilibrium $O_2^{16} + O_2^{18} \rightleftharpoons 2\ O^{16}O^{18}$ was established over a hot platinum wire, and by operating for eighteen months, 250 ml. of 99.5 per cent pure O_2^{18} were obtained (Clusius, Dickel and Becker, 1943). A thermal diffusion column operating for comparatively short periods of time still gives a good enrichment of O^{18} (Welles, 1946; Lauder, 1947), and this method will be used to provide highly enriched material for use in tracer work. A unique method which seems to have great potentialities uses thermal diffusion to establish a counter current flow of carbon monoxide and carbon dioxide: an exchange reaction takes place at the hot wire and enriches both C^{13} and O^{18} (Taylor and Bernstein, 1947).

O^{18} is conveniently stored and transported as heavy water, H_2O^{18}. Such water may be used directly in many experiments, and from it, labeled oxygen, carbon dioxide and many inorganic salts may be obtained. Practically no work has been reported on the preparation of organic compounds labeled with O^{18}. Enriched ethanol and methanol have been prepared in the fractionating columns used for water distillation, and H_2O^{18} should provide a useful starting point for organic syntheses. Oxygen labeled glycine has been prepared by an exchange reaction, and by the hydrolysis of amino-acetonitrile with H_2O^{18} (Bentley, 1947).

$$H_2NCH_2CN + H_2O^{18} \rightarrow H_2NCH_2CO_2{}^{18}H$$

FACTORS AFFECTING THE USE OF O^{18}

It was shown by Lewis (1933) that, after sulfur dioxide and water have been brought into equilibrium, there is a concentration of O^{18} in the sulfur dioxide. This was the first instance of a simple type of isotopic reaction:

$$SO_2{}^{16} + H_2O^{18} \rightleftharpoons SO_2{}^{18} + H_2O^{16}$$

These differences in reactivities of isotopic molecules are mainly attributed to differences in zero-point energy, and one consequence of this fact is that isotope separation by chemical reactions of this type is possible. A study of similar exchange reactions of many compounds has shown a striking lability of oxygen in a large number of inorganic and organic molecules. The oxygen exchanges of inorganic anions and some organic compounds have been summarized (Reitz, 1939; Bentley, 1948a). Before using O^{18} as a tracer it is necessary to be sure that simple exchange reactions are not taking place which would invalidate the results; alternatively, if exchange reactions do take place, it is often possible to make appropriate corrections.

The only inorganic anions which are known not to

exchange oxygen with H_2O^{18} under any conditions are nitrite, nitrate, phosphate, sulfate, selenate, chlorate and perchlorate. Anion exchange reactions are considered to take place through the initial formation of undissociated acid by hydrolysis, followed by reversible anhydride formation. Confirmation of this theory is provided by the catalytic effects (positive or negative) of acids and alkalies on the exchange reactions. Thus for example, sulfuric acid undergoes exchange on long heating with H_2O^{18}:

$$H_2SO_4 \rightleftharpoons SO_3 + H_2O$$
$$SO_3 + H_2O^{18} \rightleftharpoons H_2SO_4{}^{18}$$

The relative slowness of this exchange (compared, for example, with carbon dioxide) is due to the small amount of anhydride in solution (Hyde, 1941). Salts of sulfuric acid do not exchange, and a heavy sodium sulfate has been used in metabolic studies (Aten and Hevesy, 1938).

Of special importance is the exchange reaction between carbon dioxide, its salts and water. When carbon dioxide is equilibrated with H_2O^{18}, the O^{18} is concentrated in the carbon dioxide. The reactions involved, and the pertinent data are given in Table 2. Because the carbon dioxide must come to equilibrium with all of the water, the exchange takes place slowly, compared with the time needed to

TABLE 2. EXCHANGE REACTIONS OF CARBON DIOXIDE

	K at 298°A
$CO_2{}^{16}+2H_2O^{18}(l)=CO_2{}^{18}+2H_2O^{16}(l)$	1.080*
$CO_2{}^{16}+CO_2{}^{18} \quad =2CO^{16}O^{18}$	3.993*
$CO_2{}^{16}+H_2O^{18}(l) \quad =CO^{16}O^{18}+H_2O^{16}(l)$	2.076†

* Urey and Greiff, 1935.
† Mears and Sobotka, 1939.

establish the chemical equilibrium (elementic equilibrium). The equilibration is completed more quickly by shaking, warming or in the presence of catalysts, particularly carbonic anhydrase. In some experiments the rate constant was increased 4,000 fold by addition of one milligram of pure carbonic anhydrase to one liter of solution (Lounsbury, 1947). Sodium bicarbonate at pH 8 exchanges oxygen by simple hydration.

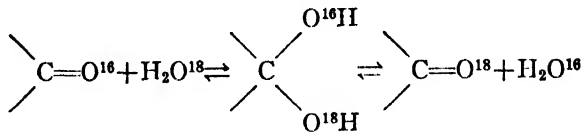
$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

All the oxygen atoms of sodium carbonate exchange, but more quickly than would be expected from simple hydration. The time of half exchange at 25° is 28 hours, and in solution at pH 10, reaction takes place through the bicarbonate ion.

$$CO_2 + OH^- \rightleftharpoons HCO_3^-$$

As, however, the concentration of hydroxyl ions is further increased, the exchange becomes very much diminished due to the extremely small amount of carbon dioxide present, and is completely inhibited in the presence of 0.04M NaOH (Mills and Urey, 1940).

Of the organic compounds which have been studied, many carbonyl compounds undergo exchange by reversible hydration to the diol.



As examples, there are the complete and rapid exchanges of aldehydes (such as acetaldehyde and benzaldehyde), the partial exchange of many sugars where only one oxygen atom is replaced, and the exchange of carboxylic acids. Interesting results have been obtained from a study of the simultaneous mutarotation and exchange of glucose and fructose (Goto and Titani, 1941). At 100°, the speed of the two reactions is roughly parallel, but at lower temperatures, mutarotation takes place without exchange. It is therefore considered that at room temperature, mutarotation is a stereochemical rearrangement involving hydrogen transfer, but that at higher temperatures the mutarotation may proceed partly or wholly through an intermediate hydrated compound. As with inorganic compounds, the extent of exchange is often very dependent on the pH of the reaction mixture. In the series acetic acid, monochloracetic acid and trichloracetic acid, with increasing acid strength the exchange takes place with increased rapidity. Alcoholic and phenolic hydroxyl groups do not exchange oxygen atoms with water, unless there are special factors leading to labilization as in the case of the acid catalyzed exchange of trianisyl alcohol.

Attempts have been made to profit from such exchange reactions. The exchange of some proteins with H_2O^{18} was studied to obtain information about their structure (Mears and Sobotka, 1939). Of the oxygenated groups present, only the carboxyl groups exchanged, particularly at low pH values. There was no exchange with dried egg albumin, but with pepsin at pH 4.0, a 13 percent exchange took place, corresponding to the oxygen present in the free carboxyl groups of the dicarboxylic amino-acids.

ANALYTICAL METHODS

The analysis of O^{18} is carried out, either by a density determination on a water sample, or by the use of a mass spectrometer. Both methods have their peculiar advantages: density may be determined to better than 0.05γ (1γ is 1 part per million and an excess density of 1γ corresponds to 0.0008 atom per cent excess), but relatively large samples are needed, and very careful purification is necessary. The mass spectrometer does not need such carefully purified samples and can be operated with very small samples. Gases such as oxygen and carbon dioxide can be analyzed directly, and an accuracy of 0.5 to 1.0γ is possible with a good instrument.

Densimetric Methods. The compound to be analyzed is converted to water, usually by a reduction process with oxygen free hydrogen. To avoid errors due to a possible concentration of deuterium, the water should be electrolysed and the oxygen obtained recombined with normal hydrogen (Datta, Day and Ingold, 1937). After vigorous purification, the density is determined by one of the many methods available. The method most generally used depends on the Cartesian Diver principle, and several modifications are available (*e.g.*, Rittenberg and Schoenheimer, 1935; Emeléus, James, King, Pearson, Purcell and Briscoe, 1934).

Mass Spectrometric Analysis. Several gases may be used in the mass spectrometer, but owing to the persistent water "background," present in almost all spectrometers, it is not possible to analyze water directly. Oxygen may be used, but it is not too satisfactory owing to its action on the spectrometer filament. The most convenient gas is certainly carbon dioxide; it may be obtained from many carboxylic acids by simple decarboxylation, and its utility is increased since water samples may be analyzed by equilibration with carbon dioxide. The use of the exchange reaction

$$CO_2^{16}\ (\text{gas}) + H_2O^{18}\ (\text{liq.}) \rightleftharpoons CO^{16}O^{18}\ (\text{gas}) + H_2O^{16}\ (\text{liq.})$$

for the analysis of water was due to Cohn and Urey, 1938). Originally, a known weight of water was mixed with carbon dioxide at atmospheric pressure and stood for some hours so that the equilibrium was completely established. Equilibration is completed more rapidly by the addition of a little carbonic anhydrase, and, using a tube of small volume, as little as five mg. of water may be analyzed. In some cases it is convenient to carry out the equilibration by standing the water with sodium bicarbonate, carbonic anhydrase again being a useful catalyst. If R is the abundance ratio (44:46) of the carbon dioxide after equilibration, consideration of the equilibrium equation shows that,

$$\text{Atom \% } O^{18} \text{ in water} = \frac{100}{K/2\ (2R - 1) + 1}$$

where K is the equilibrium constant (2.076) of the reaction (Bentley, 1948a).

A general method for the analysis of O^{18} in small amounts of organic compounds has been lacking, but a suitable apparatus has now been devised. This is a simplification and modification of the ter Meulen method for non-isotopic oxygen analysis. The compound is quickly decomposed in a stream of pure, dry hydrogen, over a platinum spiral, and the mixed vapors reduced to methane and water over a thoria promoted nickel catalyst (Russell and Fulton, 1933). The water is frozen in a U-tube, which, provided with a suitable stopper and stock cock, becomes the tube in which the water is subsequently equilibrated with carbon dioxide. Equilibration is catalyzed with carbonic anhydrase, and the analysis is quite rapid. The amount of material required varies with its oxygen content, but sufficient should be converted to yield about five mg. of water (*e.g.* with O^{18} glycine, about 10-15 mg. are taken).

The Uses of O^{18}

Apart from the exchange reactions which have been discussed previously, the first use of O^{18} as a tracer was by Polanyi and Szabo in their classical study of saponification (1934). This was one of the earliest uses of a stable isotope in organic or biochemical research, and pointed the way to the sphere in which O^{18} is particularly valuable—the study of reaction mechanisms. The retention of optical activity during hydrolysis of esters made from optically active alcohols had suggested that alkaline hydrolysis took place with splitting of the bond between oxygen and the carbonyl group. Conclusive proof of this was provided by the alkaline hydrolysis of *n*-amyl acetate in H_2O^{18}. The amyl alcohol produced was dehydrated by repeated circulation over bauxite at 400°, and the water subjected to vigorous purification. Its density was shown to be less than that of the water used for hydrolysis, so that the hydrolytic split must have taken place as follows:

$$CH_3C(=O)\text{—}O\text{—}C_5H_{11} \rightarrow CH_3CO_2{}^{18}H + C_5H_{11}OH$$
$$HO^{18}\text{—}H$$

Acid catalyzed hydrolysis in H_2O^{18} followed the same course; using methyl hydrogen succinate it was demonstrated that O^{18} did not enter the methanol molecule (Datta, Day and Ingold, 1939).

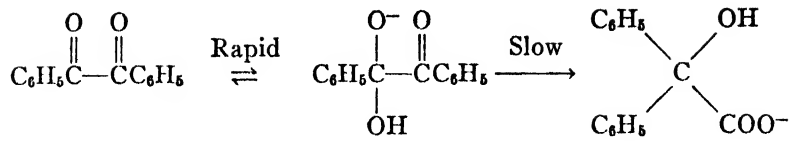
Esterification was also studied with O^{18} as a tracer, and for the first time a clear picture of the nature of esterification and hydrolysis was obtained. Methanol containing excess O^{18} (obtained from the Pegram column) was used to esterify benzoic acid, and the water produced contained only the normal abundance of O^{18} (Roberts and Urey, 1939). The rate of simple exchange of benzoic acid was known to be too slow to affect the reaction and so esterification was represented by the following equation:

$$CH_3O^{18}\text{—}\boxed{H + HO}\text{—}OC\cdot C_6H_5 \rightarrow H_2O + CH_3O^{18}OC\cdot C_6H_5$$

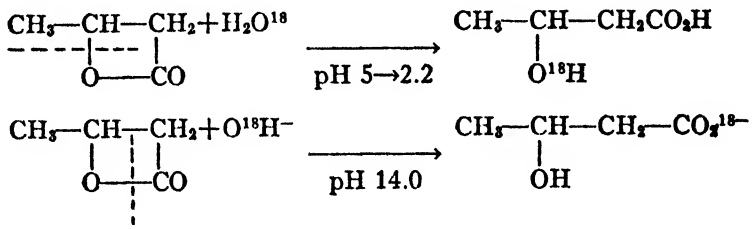
It would probably be of interest to investigate the effect of substituent groups on these processes.

Many other reactions have been studied with O^{18} and for convenience these will be briefly summarized. *Benzilic Acid Rearrangement.* (Roberts and Urey, 1938).

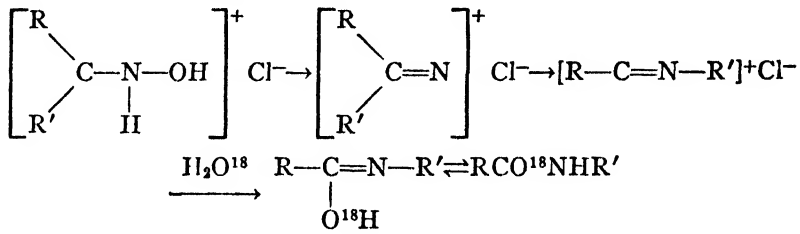
Benzil exchanged O^{18} more rapidly in alkaline than in neutral solution; it was concluded that the first stage was the rapid reversible addition of OH^- to benzil, followed by a slow and rate controlling rearrangement of the ion.



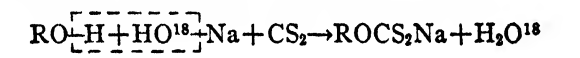
Lactone Hydrolysis (Olson and Hyde, 1941.)
Hydrolysis of β-butyrolactone took place by different mechanisms at different pH conditions.



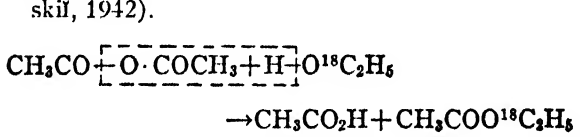
Beckmann Rearrangement. (Brodskiĭ and Miklukhin, 1941).
The mechanism proposed by Stieglitz was confirmed.



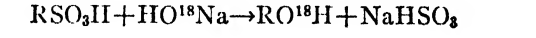
Xanthation of Alcohols. (Makolkin, 1942a).



Acylation with Anhydrides. (Dedusenko and Brodskiĭ, 1942).

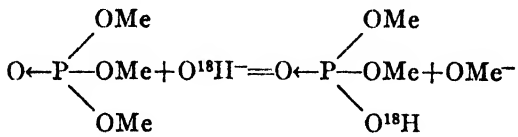


Alkali Fusion. (Makolkin, 1942b).

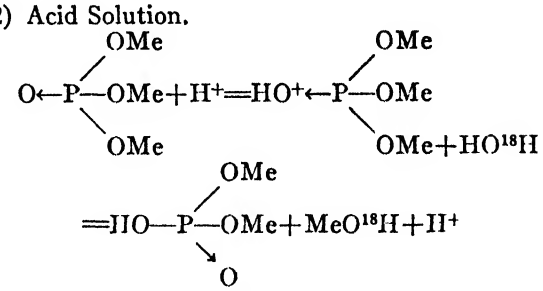


Phosphate Ester Hydrolysis. (Blumenthal and Herbert, 1945).
Different hydrolytic mechanisms under acid or alkaline conditions.

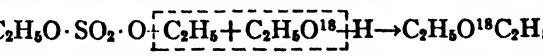
1) Alkaline Solution.



2) Acid Solution.



Ether Synthesis. (Lauder and Green, 1946).



More recently, the hydrolysis of acetyl phosphate has been studied with H_2O^{18}, and as these results are of biochemical interest they will be considered in more detail. Acetyl phosphate, first characterized as a bacterial metabolite (Lipmann, 1944), assumed increasing importance, especially when the incorporation of acetic acid into compounds such as fatty acids, sterols and hemin was demonstrated by isotope methods (Bloch and Rittenberg, 1942, 1945). Experiments with bacterial enzymes showed that the phosphate group could be transferred to several compounds and a similar transfer has now been demonstrated with pigeon liver extracts (Kaplan and Lipmann, 1948).

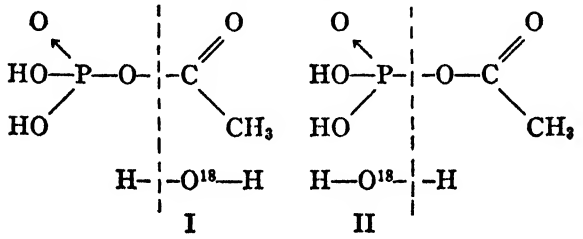
As a mixed anhydride, it was expected that acetyl phosphate would act as an acetylating agent. The

use of phosphoric acid as a catalyst in some acetylations with acetic anhydride is of interest in this connection. In a number of isotope studies with the enzyme system of *E. coli* a reversal of the initial phosphoroclastic reaction was observed, and it was concluded that formate could be acetylated by acetyl phosphate.

$$HCOOH + CH_3COOPO_3H_2 \rightleftharpoons CH_3COCOOH + H_3PO_4$$

Later work, however, with a double tagging technique apparently contradicts these results, and it has been shown that formate is fixed in pyruvate without the formation of acetate or acetyl phosphate as essential intermediates (Strecker, Krampitz and Wood, 1948).

The supposition that acetyl phosphate may act either as an acetylating or phosphorylating agent requires that the anhydride bond can be split in two different ways. The mechanism of its hydrolysis, was therefore studied by the use of H_2O^{18}. In an acetylating process the C — O bond would split (I), O^{18} being incorporated into the acetate; in a phosphorylating process the P — O bond would split leading to O^{18} in the phosphate (II).



I II

It has been shown that, contrary to early reports, phosphate does not exchange O^{18} (Winter, Carlton and Briscoe, 1940). As however, an analytical method for estimation of O^{18} in phosphate was not readily available, only the acetate portion of the molecule was studied. A slow exchange with acetate had been observed in the presence of hydrochloric acid (Roberts, 1938), and this exchange was studied under conditions used in the hydrolysis, i.e. in stoppered Pyrex tubes without exclusion of atmospheric carbon dioxide. Agreeing with the latter author, a slow exchange was observed at room temperature, increasing with increased temperature, and especially catalyzed by strong acid. In the presence of alkali, even on warming, there was only a slight exchange. (See Table 3.)

In these experiments, after carrying out the exchange or the hydrolysis, the acetic acid was rapidly distilled at low temperature, neutralized with ammonia, and converted to silver acetate. This was decarboxylated by heating *in vacuo,* and the carbon dioxide obtained was analyzed in the spectrometer. The acetyl phosphate was a synthetic material, prepared by the action of ketene on syrupy phosphoric acid in ethereal solution (Bentley, 1948b). When silver acetyl phosphate was hydrolyzed in H_2O^{18} (1.03 atom % excess) in the presence of potassium hydroxide, under conditions, that is, where the exchange reaction would not take place, O^{18} was found in the acetic acid isolated at the end of the hydrolysis. If the split takes place as in I, then 50 percent of the atoms present in the acetic acid would be derived from the water. With acetyl phosphate and dibenzyl acetyl phosphate, the experimental data (Table 4) show that this value was closely attained.

TABLE 3. EXCHANGE OF O ATOMS OF ACETIC ACID

Reagent	Time, hr.	Temp., °C.	Atom % excess of water used	Atom % excess of isolated Ag Ac	% Exchange
Acetic Acid*	1	25	1.03	0.012	1.2
Acetic Acid*	16	25	1.03	0.047	4.6
Acetic Acid*	62	25	1.03	0.129	12.5
Acetic Acid*	3	100	1.03	0.866	87.4
NaAc+HCl†	6	100	1.03	0.765	91.6
NaAc+$Ba(OH)_2$‡	6	100	1.03	0.056	5.4

Correction has been made for the dilution of the H_2O^{18} by the normal oxygen atoms of the acetic acid.

* 1 mm. per 1 ml.

† Sodium acetate, 1 mm.; H_2O^{18}, 0.8 ml.; 12N HCl, 0.2 ml. Further correction made for dilution by the normal oxygen atoms in aqueous HCl.

‡ Sodium acetate, 1 mm.; H_2O^{18}, 1.0 ml.; anhyd. $Ba(OH)_2$, 0.25 mm.

TABLE 4. ALKALINE HYDROLYSIS OF ACETYL PHOSPHATE

Reagent	Atom % excess of water used	Atom % excess of isolated Ag Ac	% O atoms derived from water
Silver acetyl phosphate+KOH	1.03	0.453	45.3
Dibenzyl acetyl phosphate+KOH	1.03	0.450	45.7

Correction has been made for dilution of the H_2O^{18} by the normal oxygen atoms of the KOH.

When salts of acetyl phosphoric acid were hydrolyzed by water, without the addition of other reagents, the solutions became acid, the pH finally obtained depending on whether the silver or sodium salt was used. Under these conditions therefore, there would be O^{18} in the acetic acid due to the simple exchange reaction. If the split took place as in the previous case, there would therefore be more than 50 percent of the oxygen atoms derived from the water. In a number of experiments (Table 5) the percentage of such oxygen atoms varied from 7.0 to a maximum of 32.7, and was always less than 50. It was concluded therefore, that the phosphorus-oxygen bond was split (II). More conclusive proof that this was the case was provided by using a preparation of the enzyme, acetyl phosphatase (kindly supplied by Dr. Lipmann). With this enzyme a rapid hydrolysis at 37° was possible, and the exchange reaction was therefore very considerably reduced. Using lithium acetyl phosphate in a slightly acid solution only a negligible amount of O^{18} was present in the acetic acid subsequently isolated.

These experiments proved that, depending on the pH, acetyl phosphate could hydrolyze by splitting the C — O or the P — O bond. In some *in vitro* experiments, acetyl phosphate has been shown to act as an acetylating agent, but no evidence has yet been obtained for non enzymatic phosphorylation reactions.

Table 5. Hydrolysis of Acetyl Phosphate under Acid Conditions in H_2O^{18}

Reagent	Time, hr.	Temp. °C.	Atom % excess of water used	Atom % excess of isolated AgAc	% O atoms derived from water
Disilver acetyl phosphate	2	85	1.03	0.300	29.1
Disilver acetyl phosphate	72	25	1.03	0 337	32.7
Disodium acetyl phosphate	2	85	1.03	0.078	7.6
Disodium acetyl phosphate	16	25	1.03	0.072	7.0
Disodium acetyl phosphate	72	25	1.03	0.088	8 5

The Elementary Analysis of Oxygen by Isotope Dilution

The use of the isotope dilution method (and its various modifications) in the analysis of complex mixtures is well established, and the method has now been extended to the elementary analysis of oxygen, carbon and nitrogen (Grosse, Hindin and Kirshenbaum, 1946). This new method is of especial value for oxygen, since no convenient method exists for its determination. The method of ter Meulen is tedious and oxygen determinations are almost always made "by difference." For the isotope dilution method, a known weight of sample, a, (from 20-40 mg.) is equilibrated with a known weight, b, of O_2^{18} (obtained by micro-electrolysis of H_2O^{18}). The equilibration is carried out in a platinum tube at 800°: carbon dioxide is rapidly isolated from the resulting mixture, and analyzed in a mass spectrometer. Complete combustion is not necessary for equilibration, and equilibrium is properly attained even if insufficient oxygen is used. If oxygen of m atom percent excess is used, and if the atom percent excess O^{18} in the equilibrated carbon dioxide is n, the weight percent of oxygen in the sample is given by

$$x = \frac{b(m - n)}{a \cdot n} \cdot 100$$

With present facilities, an accuracy of ±3 percent has been obtained, but greater accuracy will be possible with a more precise spectrometer and more highly enriched O^{18}.

The Study of Photosynthesis with O^{18}

Perhaps the most important contribution of O^{18} to biochemistry has been to provide an answer to the question: Is the oxygen evolved in the course of the photosynthetic reaction,

$$n\ CO_2 + n\ H_2O + h\nu = O_2 + (CH_2O)_n$$

derived from carbon dioxide, from water, or from both the carbon dioxide and the water? By labeling the oxygen of one or the other, it should be possible to provide a conclusive answer, and such experiments were simultaneously reported from two laboratories. In a study with active cell preparations of *Chlorella pyrenoidosa,* the photosynthetic reaction was carried out in a bicarbonate-carbonate buffer solution at pH 10 (Ruben, Randall, Kamen and Hyde, 1941). In such alkaline solutions, the previously discussed exchange reaction between carbon dioxide and water takes place rather slowly, but with a measurable velocity. Compared with the rate of photosynthesis however, it was sufficiently slow to be neglected. When the algae were grown on H_2O^{18}, the O^{18}:O^{16} ratio of the evolved oxygen (determined by a mass spectrometer) was equal to that of the enriched water; using water of normal isotopic abundance, but containing $KHCO_3^{18}$ and $K_2CO_3^{18}$, the evolved oxygen had the normal isotopic composition. It was concluded therefore that the oxygen of photosynthesis was derived from the water.

In other experiments, reported in the same year, enriched water was not used, but advantage was

taken of the very sensitive floatation method in the determination of density differences (Vinogradov and Teĭs, 1941). *Helodea Canadensis* was grown on tap water containing 0.1 percent of sodium bicarbonate; water derived from the photosynthetic oxygen showed an average density increase of 4.5γ compared with the standard Moscow River water, and water derived from the oxygen of the bicarbonate used in this experiment, an average increase of 11.6γ. The photosynthetic oxygen therefore differed from both the oxygen of the water and carbon dioxide, but approximated more closely to the water.

These experiments indicated that the photosynthetic oxygen was derived from the water. In the former experiments, this conclusion was dependent on the assumption that there was no velocity increase of the carbon dioxide-water exchange reaction inside the cells, relative to the external medium. The internal vacuoles of many plant cells are at a pH of about 6.0, and under these conditions the exchange reaction is much more rapid. In further experiments, similar to those of the Russian workers, such possibilities were avoided (Dole and Jenks, 1944). Water of normal isotopic composition was first equilibrated with carbon dioxide, using carbonic anhydrase to ensure complete equilibration. When Chlorella suspensions were grown in this equilibrated water, water prepared from the evolved oxygen had almost the same density as that of the equilibrated water (but was about 1.2γ higher) and significantly less than water made from the carbon dioxide. (Corrections were applied for an initial dilution of the sample to give a sufficiently large bulk for accurate measurement, and for the isotopic composition of the hydrogen used in preparation of the water samples.) A recent report on work with O^{18} at McMaster University has confirmed these findings for land plants. The interesting observation has also been made that with leaves in an atmosphere of $CO_2{}^{18}$, the O^{18} is incorporated into the tissue more rapidly in the daylight than in the dark (Lounsbury, 1947).

It has been suggested that the slightly increased O^{18} content of photosynthetic oxygen above that of normal water is due to the exchange reaction of oxygen with water.

$$O_2{}^{16}\ (\text{gas}) + 2H_2O^{18}\ (\text{liq.}) = O_2{}^{18}\ (\text{gas}) + 2H_2O^{16}\ (\text{liq.})$$

The observed increase closely approaches that required on the basis of an enrichment factor of 1.010 at 273.1° A (Urey and Greiff, 1935). In the work by Ruben and his co-workers, no evidence was obtained for an exchange of photosynthetic oxygen in presence of oxygen, or oxygen used in respiration: their mass spectrometric determinations were probably not accurate to 1γ, so the evidence, while not conclusive, suggests that Chlorella catalyzes the equilibrium reaction between oxygen and water.

A concentration of O^{18} in plant tissue would be expected since: 1) water is apparently decomposed to oxygen; 2) the oxygen of carbon dioxide is about 8γ heavier than that of fresh water. Such a concentration has apparently been observed in carbohydrates. Sugar, burnt in electrolytic oxygen, gave water of Δ d about 5γ (Morita and Titani, 1936).

The Dole Effect

Research on the isotopic composition of oxygen derived from various natural sources was stimulated by the discovery that water prepared from atmospheric oxygen and normal hydrogen had a greater density than fresh water (Dole, 1935). This observation was quickly confirmed and the phenomenon is known as the Dole Effect; its discovery and the work to be subsequently discussed, was only made possible by the very accurate methods available for determining differences in density. These methods are accurate to better than ±0.05γ unit and the limiting factor is rather the difficulty of purifying the water sample. An independent method for measuring the Dole Effect, which utilizes the exchange reaction of an inorganic anion has also been described (Alexander and Hall, 1940).

The discovery of the Dole Effect has aroused discussion about the origin of atmospheric oxygen. Of greatest interest to the biologist, is the fact that while it is generally agreed that atmospheric oxygen has been, and is being, predominantly formed by photosynthesis, yet the oxygen of the atmosphere contains more O^{18} than fresh waters—from which as was shown earlier, photosynthetic oxygen is almost certainly derived. Before this point is considered in more detail, the rather wide differences in isotopic abundance of various oxygens will be discussed; as usual, these differences will be given in terms of the difference in density, Δ d, (in γ units) of the water produced on combination of the oxygen with normal hydrogen. Taking fresh water as a standard (there are only rather small variations in the densities of various fresh waters not subject to special influences) the following generalizations may be made.

A) Rocks and natural ores, not containing carbonates, have the same O^{18} content as fresh water (Hall and Hochanadel, 1940).

B) Sea water is more dense than fresh water, having Δ d about 2γ (Gilfillan, 1934; Wirth, Thomson, and Utterback, 1935; Kassatkina and Florenskiĭ, 1941). This value is in agreement with the density increase to be expected from the evaporation of water.

C) Water from the oxygen of carbonate rocks has Δ d, 8γ (and there is no significant difference in isotopic composition as a function of age) (Dole and Slobod, 1940). There is good agreement between the calculated and observed fractionation expected from the exchange reaction:

$$H_2O^{18} + 1/3\ CO_3{}^{16=} \rightleftharpoons H_2O^{16} + 1/3\ CO_3{}^{18=}$$

It indicates the formation of such rocks from carbon dioxide in sea water, the carbon dioxide being previously enriched by the exchange reaction.

D) In addition to these major variations, several other natural processes serve to fractionate the oxygen (and hydrogen) isotopes to varying degrees. During the freezing of water, O^{18} is concentrated in the solid phase, and this fractionation takes place in the formation of snow and natural ices (Teĭs and Florenskiĭ, 1941). Water basins fed by melting snow or glaciers therefore often show an increased O^{18} content.

These differences find a satisfactory explanation in terms of known isotope fractionating processes, but the greater proportion of O^{18} in the atmosphere (relative to fresh water) is apparently in contradiction to the supposition that atmospheric oxygen has largely been formed by photosynthesis, and therefore from water. (The study of photosynthesis by O^{18} has so far been limited to fresh water plants, but it seems unlikely that any profound difference will be found in marine plants.) The results with Helodea suggest that the density of the water from photosynthetic oxygen is sufficiently greater than that of the nutrient water to account for most of the effect. These results are given in Table 6; while most of oxygen must have been derived from the water, it is nevertheless 4.5γ units heavier. The rest of the Dole Effect could be due to a contribution of 2γ from concentration of O^{18} in sea water and 1γ from the exchange reaction between oxygen and water (discussed below).

TABLE 6. PHOTOSYNTHESIS WITH *Helodea Canadensis* (Vinogradov and Teĭs, 1941)

	Δd
Water from photosynthetic oxygen	+ 4.5γ
Water from atmospheric oxygen	+ 7.3γ
Water from $NaHCO_3$	+11.6γ

All referred to Moscow River water.

These results are apparently at variance with those of the American workers, and until more experimental work is available it is not possible to assume that the Dole Effect is to be explained entirely by the distribution of O^{18} during photosynthesis. Part of the Dole Effect may be due to the exchange reactions between oxygen and water.

$$O_2^{16}\ (gas) + 2H_2O^{18}\ (gas) \rightleftharpoons O_2^{18}\ (gas) + 2H_2O^{16}\ (gas)$$

Enrichment factor at 298° A, = 1.020 I.

$$O_2^{16}\ (gas) + 2H_2O^{18}\ (liq.) \rightleftharpoons O_2^{18}\ (gas) + 2H_2O^{16}\ (liq.)$$

Enrichment factor at 298° A, = 1.006. II.

Reaction I has been studied experimentally, and takes place over platinum at high temperatures: below 500° the extent of exchange is slight (Morita and Titani, 1937). Slight enrichment apparently due to reaction II was observed in the Chlorella experiments of Dole and Jenks (1944). The enrichment to be expected from either reaction is still insufficient to account for the Dole Effect. The possibility has been investigated that the enhanced O^{18} content of the atmosphere might result from a more rapid utilization of O^{16} by soil bacteria (Dole, Hawkings and Barker, 1947). Such bacteria were grown aerobically on a synthetic culture medium in a series of ten flasks, and the residual oxygen accumulated in the tenth flask was analyzed isotopically. Water prepared from it had an excess density of 1.5—0.5γ. The single stage fractionation factor calculated from these results falls considerably short of the value required to account for the Dole Effect. If there are mechanisms, as yet unexplored, by which oxygen atoms could exchange between carbon dioxide and oxygen in the atmosphere, the Dole Effect could probably be accounted for entirely in terms of physical processes.

In the 1947 Liversidge Lecture to the Chemical Society, Urey pointed out that calculation of the equilibrium constants for the exchange reaction

$$H_2O^{18} + 1/3\ CO_3^{16=} \rightleftharpoons H_2O^{16} + 1/3\ CO_3^{18=}$$

was of particular interest, and would lead to interesting results. A change from 0° to 25° should change the O^{18} content of carbonates by 1.004 relative to liquid water, and accurate determinations of the O^{18} content of carbonate rocks could therefore be used to determine the temperature at which they were formed. Urey estimates that with a precision mass spectrometer, determining the abundance ratio to ± 0.001, it would be possible to determine the temperature of deposition of carbonate rocks with an error of only 6° C. Since sea water contains more O^{18} than fresh water, carbonates deposited in sea water should contain more O^{18} than those deposited in fresh water. It should therefore also be possible to distinguish between carbonates deposited in sea water and fresh water. Other oxy salts show similar temperature coefficients to that of carbonate, and should be available for similar studies (Urey, 1947).

O^{18} IN METABOLIC STUDIES

Apart from the photosynthetic studies, only two instances of the use of O^{18} in the study of animal metabolism have been reported. This has been partly due to the relative unavailability of the isotope, and partly to the experimental difficulties.

In 1938, the fate of the sulfate radical in the animal was investigated (Aten and Hevesy, 1938); a heavy oxygen sodium sulfate was prepared from sulfuryl chloride and H_2O^{18}. After solution in water of normal isotopic composition it was injected into a rabbit, and sulfate recovered from the urine was analyzed for O^{18} (reduction with purified carbon, and reduction of these gases to water with hydro-

gen). The sulfate contained a considerable amount of O^{18} and after allowing for the ordinary sulfate excreted, it was concluded that most of the sulfate had passed unchanged through the body. At most, only a small fraction of the injected sulfate ions could have exchanged with those already present.

The fate of respiratory oxygen is a matter of some interest, and it seems surprising that only one attempt has been made to study this problem with O^{18}. Two possibilities have been considered: inspired oxygen enters directly into the oxidation of carbon compounds; or else inspired oxygen combines first with the hydrogen of water, the original oxygen of the latter then being expired as carbon dioxide. In the experiment of Day and Sheel (1938), rats were kept in an atmosphere enriched with O^{18} (equivalent to Δ d, + 300γ). The expired carbon dioxide gave water of excess density, Δ d = + 40γ, and from this result it was concluded that the former alternative was correct. This conclusion, however, was not justified, since the equilibrating effect of carbonic anhydrase was not taken into account. The excess density could have been due to the initial production of either a heavy water or a heavy carbon dioxide, followed by subsequent equilibration. More information could possibly be obtained by studying the O^{18} content of expired carbon dioxide after allowing an animal to drink H_2O^{18}, or by taking advantage of the small density differences between fresh water and atmospheric oxygen.

Allied with this problem is the function of hydrogen peroxide in metabolism, and some preliminary studies on the mechanism of its decomposition by catalase have been carried out with the help of O^{18}. The decomposition of hydrogen peroxide by reagents such as ferrous sulfate and colloidal metals is a chain reaction in which free hydroxyl and peroxide radicals take part (Haber and Weiss, 1934). The decomposition by catalase is certainly a more complex process; it is known that a comparatively stable compound is formed between one mole of hydrogen peroxide and one mole of catalase, and it has also been suggested that a chain reaction may be involved in the decomposition. Since free radicals have been considered to be involved in some enzymic processes, it was of interest to see whether they could be detected in the decomposition of peroxide by catalase. The reaction has therefore been investigated in H_2O^{18}. It was assumed that if free hydroxyl radicals were formed, they would undergo a ready exchange with water molecules (*e.g.* Waters, 1946).

$$HO.^{16} + HO^{18}H \rightleftharpoons HO^{16}H + .O^{18}H$$

The labeled radicals would then take part in the chain reaction and several reactions could lead to the production of O^{18} enriched oxygen from the hydroxyl radicals. Using water of 1.6 atom percent excess no O^{18} was found in the liberated oxygen, and a similar result was obtained when the peroxide was decomposed with ferrous sulfate. Since free hydroxyl radicals are almost certainly formed in this reaction, it seems likely that solvent water molecules are not involved in the decomposition, or that the exchange of hydroxyl radicals with water takes place less readily then has been supposed. The experiments will be repeated when more highly concentrated O^{18} is available since with the 1.6 percent water only rather small dilutions can be detected.

An alternative method is now being used to try to find out whether the decomposition of hydrogen peroxide with catalase is a chain reaction. It has been shown that hydroxyl radicals can initiate the chain polymerization of substances such as acrylonitrile and methyl acrylate (Baxendale, Evans and Park, 1946). If a mixture of hydrogen peroxide and catalase is shown to bring about a similar polymerization, it would be good evidence for the production of the hydroxyl radical during the decomposition.

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Discussion

LIPMANN: Dr. Bentley's differentiation of the acetyl or phosphoryl split of acetyl phosphate in alkali or acid respectively appears of considerable biochemical significance. Acetyl phosphate or analogous compounds react enzymatically as acyl donors as well as phosphate donors. *Synthetic* acetyl phosphate has been found by ourselves and others to react as an excellent phosphate donor in a great variety of enzymatic reactions. But it does not react as acetyl donor. Nevertheless a "natural" acetyl phosphate formed by enzymatic reaction between acetate and ATP serves easily as acetyl donor. Modifications of acyl phosphates appear therefore to fulfill metabolically two functions, (1) to deliver phosphate into the metabolic pool of energy-rich phosphate and (2) to donate their activated acyl moiety for synthesis. I wonder if a discussion of the respective mechanisms of acid and alkali, *i.e.*, phosphate and acyl, split of acyl phosphate may not prove helpful for an understanding of the physiological double function of these compounds.

EXPERIMENTS WITH N^{15} ON PURINES FROM NUCLEI AND CYTOPLASM OF NORMAL AND REGENERATING LIVER

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In experiments with radioactive phosphorus on the turnover of polynucleotides in rat liver, Brues and his group (Brues, Tracy and Cohn, 1944) have demonstrated a high stability of polydesoxyribonucleotide (DNA) and a comparatively rapid turnover of polyribosenucleotides (PNA) during normal conditions, that is, non-growth in the adult rat. During regeneration following partial hepatectomy the turnover of both polynucleotides increased. In fractions from isolated cell nuclei the concentration of radioactive phosphorus was somewhat higher than that in DNA from non-growing and regenerating whole liver. The authors conclude that a part of the radioactive phosphorus taken up by nuclear fractions may represent a small, very labile moiety, as suggested by Marshak's observation (1941) that considerable P^{32} appears in nuclear protein within an hour. The interpretation of these experiments is complicated by the fact that the polynucleotides from nuclei were not separated from each other.

By fractionation of extractable polynucleotides (Hammarsten, 1947) on normal and regenerating liver provided for us by Stowell (1948), we found that the more rapid increase of polynucleotides per dry weight (chiefly of PNA) occurred during the first 24 hours after removal of about 65 percent of the liver (Table 1).

This finding is in agreement with numerous cytological observations by Caspersson and his coworkers (1947) on early stages of cell activity. The correlation of the formation of nucleic acid and protein has been discussed by them especially in connection with nuclear activity.

The determinations of turnover rates by Brues and his group were made on the fourth to the 17th day after hepatectomy. It seemed to us that earlier stages of regeneration should offer favorable conditions for a biochemical study of the assumed correlation between the formation of nucleic acids and proteins.

In the following paper results will be presented upon the nitrogen turnover in purines and protein fractions from cell nuclei and cytoplasm in non-growing and regenerating liver from the adult rat. Isotopic (N^{15}) glycine has been administered to normal rats and to hepatectomized rats 16 and 22 hours after the operation. The animals were sacrificed six hours after the last injection of isotopic glycine (28 hours after the operation).

TABLE 1. DETERMINATIONS OF EXTRACTED POLYNUCLEOTIDES IN NON-GROWING AND REGENERATING LIVER FROM RAT
The numbers represent mg. phosphorus (P) and nitrogen (N) per 100 g. dry weight

Organ	Time in hours after partial hepatectomy	PNA+DNA		PNA		DNA	PNA / DNA
		P	N	P	N	P	
Non-growing liver	0	318	540	239	430	81	2.9
Regenerating liver	12	416	720	319	—	97	3.3
	24	459	820	326	--	106	3.1
	42	475	820	338	—	102	3.3

The experimental evidence suggests that at this stage of regeneration the formation of purines in polyribosenucleotides in cell nuclei may be independent of the formation of proteins in the nuclei.

EXPERIMENTAL DATA

Partial hepatectomy. Adult albino rats of about 150 to 250 g. in weight were employed. The rats were taken in the morning from the animal house without previous withdrawal of food. After moderate ether anesthesia (administered in a glass jar) the rat was tied to a small table and the anesthesia continued with a small amount of trichloroethylene, if necessary. The skin was divided from left to right and the abdomen opened by a three to four cm. incision from the xiphoid process caudally in the linea alba. The median lobes were readily delivered and the pedicle tied. Thereafter the left lateral lobe was pressed out through the opening, isolated and the pedicle tied. Both lobes were excised and the blood drained on filter paper. The abdomen was closed and at the same time the anesthesia was

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stopped, so that the rat was usually sitting up within a few minutes. No aseptic precautions were taken. Thus the whole procedure could easily be performed within 10 to 15 minutes by one person. There was no mortality.

Thus our procedure is essentially in accordance with those earlier described (*e.g.* by Crandall and Drabkin, 1946, or by Brues, Drury and Brues, 1936). The residual liver, however, was found to vary between 37 and 81 percent of the weight of the excised liver (extirpate), the mean value of 17 experiments being 60 with a spread of 12 percent. The residual liver has been calculated as 60 percent of the weight of the extirpate in order to give a rough estimate of the regeneration. With one exception (rat 190) all rats showed a regeneration of from 11 to 86 percent of the calculated residue with a mean figure of 48 percent in 28 hours. The deviations from the experiences of other investigators (*loc. cit.*) may be due to the relative nonhomogeneity of our material and in part to the modified technique.

In order to check the cellular regeneration the frequency of mitoses in normal and regenerating livers was determined, as described by Brues and Marble. In 5μ slices stained with Ehrlich hematoxylin and eosin about 1000 cells were counted. Only fully developed monasters and diasters were counted. The results are in accordance with earlier investigations and appear in Table 2.

TABLE 2

	Number of animals	Mean values			Limits % mitoses
		Number of cells	Number of mitoses	% mitoses	
Controls					
Normal liver	5	937	0	0	0
Regenerating liver	17	1097	13.9	1.2	.4–2.3
Experiments with isotopic glycine					
Normal liver	4	1004	(0.25)	(0.025)	–0.1
Regenerating liver	5	994	16.5	1.6	0.3–2.5

Glycine had been synthesized from ammonia containing 32 atom percent excess N^{15}, according to Schoenheimer and Ratner (1939).

Each rat received two subcutaneous injections of 50 mg. glycine per 100 g. body weight according to the schedule mentioned above. Twenty normal rats ranging from 150 to 220 g. (mean 186 g.) with a total liver weight of 136 g. received isotopic glycine in exactly the same way as the operated animals. The livers were fractionated according to Dounce's method with some modifications to preserve the brittle nuclei from the regenerating livers. The sliced organs from each group of seven rats were thrown into 100 ml. of 0.05 molar citric acid and treated at 0° C. in a blender (the Swiss type "Turmix") for 1½ min. The suspension was centrifuged in 50 ml. conical tubes (max. radial distance from center 13 cm.) in the cold in the following way (Table 3).

TABLE 3. FRACTIONATION OF LIVER TISSUE

Operation No.	Rotations per min.	Centrifuging time in min.	Sediment	Supernatant
1	1000	2	Discarded	To Operation No. 2.
2	2000	10	Suspended in 40 ml. 0.01 molar citric acid. To Operation No. 3.	Cytoplasm to Operation No. 6.
3	1500	10	Suspended as above. To Operation No. 4.	
4	1500	5	Suspended as above. To Operation No. 5.	
5	1500	5	Dried with alcohol and ether. *Cell nuclei.*	
6	3000	10	Discarded	Neutralized to pH 5. To Operation No. 7.
7	3000	10	Dried with alcohol and ether. *Cytoplasm.*	

The yields of dry cell nuclei were 670 mg. from regenerating and 950 mg. from normal livers.

A small part of each liver in both series was put in alcohol and dried with ether (Total liver, Table 4).

The polynucleotides DNA and PNA were prepared and fractionated as described by Hammarsten (1947). The amount of PNA in normal cell nuclei had been determined with the same method in other experiments and had been found to vary in different preparations between 22 and 30 percent of the total polynucleotides of the nuclei.

It is well known that rather large quantities are needed for a complete separation of adenine and guanine from each other by crystallization. We could not obtain more than some few mg. of the mixed purines from cell nuclei and therefore a method has been worked out (Edman, Hammarsten, Löw and Reichard, 1948) for the fractionation of

them. The mixed purines were dissolved in 1 N sodium hydroxide, the monomethyl ether of ethylene glycol and water-saturated butanol, and the effluent from a starch column collected in 60 min. fractions. A typical diagram is shown in Figure 1.

TABLE 4. INJECTIONS OF ISOTOPIC GLYCINE IN RATS

Fractions	Non-growing liver		Regenerating liver	
	Atom % excess N^{15}	ϵ max. μgN/ml	Atom % excess N^{15}	ϵ max. μgN/ml.
Cytoplasm				
PNA Guanine	0.09	0.147	0.97	0.157
PNA Adenine	0.04	0.170	0.43	0.177
PNA Uridine	0.21	0.316	0.55	0.309
TCA—insoluble	0.27		0.46	
Cell Nuclei				
PNA Guanine	0.16	0.152	1.35	0.160
PNA Adenine	0.12	0.176	0.67	0.173
DNA Guanine	(0.03)	(0.139)	0.68	0.158
DNA Adenine	(0.02)	(0.169)	0.33	0.183
TCA—insoluble	0.28		0.28	
Total liver				
PNA Guanine	0.08	0.157	0.95	0.151
PNA Adenine	0.07	0.177	0.48	0.186
TCA—insoluble	0.27		0.51	

ϵ max. / μgN/ml.: Determined for Guanine (max. = 248 mμ) = 0.161; Determined for Adenine (max. = 262 mμ) = 0.180; Determined for Uridine (max. = 262 mμ) = 0.332

The fractions of each purine were pooled and the residues from vacuum distillations dissolved in 1 N hydrochloric acid; the amounts were determined at 262 (Adenine) and 248 (Guanine) mμ. The quotient

$$\frac{\text{The light absorption coefficient}}{\mu\text{g. nitrogen/ml.}}$$

was determined for all fractions (Table 4, Columns 3 and 5).

Samples from the different tissues were extracted three times with hot trichloroacetic acid according to Schneider (1944). The residues (TCA-insoluble, Table 4, Columns 2 and 4) were considered as protein fractions. Of the pyrimidine ribosides, only uridine was isolated in a sufficient quantity (from cytoplasm) for identification and analysis in the spectrometer. The ribosides were prepared by hydrolysis with pyridine and separated on a starch column by partition chromatography (Reichard, 1948). The uridine (Table 4, Columns 2 and 4) is partly derived from cytidine. This was done purposely as the material was not sufficient for the preparation of both uridine and cytidine.

DISCUSSION

In the regeneration experiments the animals were sacrificed and the measurements made in a phase of increased formation of polynucleotides (Table 1). This accounts to some extent for the increase of N^{15} content in DNA and PNA (Table 4).

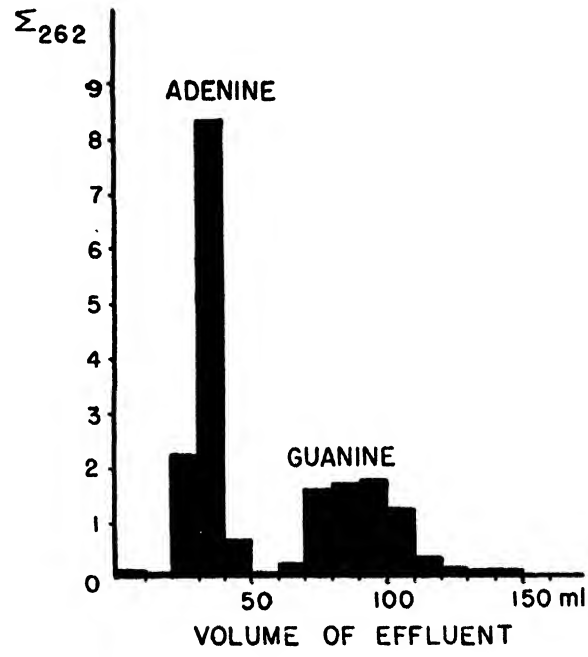

FIG. 1.

As yet the proteins have only been prepared and analyzed in the crude form of nucleotide-free TCA-precipitates. The fraction designated "cytoplasm" (Table 3) is prepared by fractionating centrifugation and may thus not necessarily contain all proteins and polynucleotides in the liver-cytoplasm as does the preparation total liver. There are certain discrepancies between the N^{15} content in corresponding nucleotide fractions from total liver and cytoplasm (Table 4), but they are not so big as to seem to affect the main results with a possible exception for the values for guanine and adenine (Column 2) DNA (cell nuclei) in non-growing liver.

The values for the TCA-insoluble fraction from total regenerating liver (Column 4) agree, however, tolerably well with the corresponding value for cytoplasm (0.51 and 0.46). Of course one might also consider the possibility that some part of the protein molecules has been split off by the action of hot trichloracetic acid.

If however the TCA-insoluble fractions are con-

sidered as representing the mixed proteins from the tissue parts from which they have been prepared, the N^{15} content (0.28) in the fraction from regenerating cell nuclei may be compared with that (0.28) in the fraction from non-growing cell nuclei. This would indicate that the protein turnover at this stage of regeneration in the cell nuclei was independent of the rapid purine turnover in nucleotides. This argument of course does not cover any TCA-soluble proteins, peptides or amino acids or a protein not using glycine in its turnover. The N^{15} content of the protein fraction from regenerating cytoplasm indicates an increase in protein formation. Guanine and adenine from the polyribosenucleotides from regenerating cell nuclei had a higher content of N^{15} (1.35 and 0.67) than the corresponding purine bases from the regenerating cytoplasm (0.97 and 0.43). The higher velocity of the nuclear fraction is in accordance with Caspersson's (1947) views. This nuclear fraction may correspond to the small and labile moiety suggested by the work of Brues *et al.* (1944) and Marshak (1941).

The higher content of N^{15} in guanine compared to adenine agrees with earlier findings of Barnes and Schoenheimer (1943) and Abrams (1948). In view of the recent demonstration by Brown, Roll, Plentl and Cavalieri (1948) that dietary adenine functions as a precursor of guanine, it is obvious that separate parts of purines and pyrimidines must be further investigated along the lines used by Sonne, Buchanan and Delluva (1946) and by Abrams, Hammarsten and Shemin (1948).

The high content of N^{15} in uridine (partly derived from cytidine) compared with that of adenine indicates that the purines were not precursors of the PNA-pyrimidines, a fact which is in accordance with Brown's demonstration that dietary adenine is not a precursor of the pyrimidines. The N^{15} values for uridine may indicate a connection between the protein and pyrimidine turnover.

Summary

Isotopic glycine (N^{15}) was administered to rats during an early stage of liver regeneration.

Guanine and adenine from the polyribosenucleotides in the cell nuclei had a higher content of N^{15} than guanine and adenine from the cytoplasm.

Uridine had a higher content of the isotope than guanine and adenine in normal cytoplasm and an N^{15} content between adenine and guanine in regenerating cytoplasm.

Protein from regenerating cell nuclei had the same content of N^{15} as protein in non-regenerating cell nuclei and non-regenerating cytoplasm. The protein in the regenerating cytoplasm had about double the content of N^{15} from normal cytoplasm.

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FORMATION OF LIPIDS BY THE MICROORGANISM *PHYCOMYCES BLAKESLEEANUS*

KARL BERNHARD

The isotope technique has proved very useful in the study of the synthetic processes in both animals and plants. The formation of lipids in animals has been studied particularly with deuterium as the tracing isotope. By this means it was demonstrated that a very rapid turnover of the fatty acids takes place in animals, particularly in organs like the liver and intestine. These and similar results led Schoenheimer to formulate the fundamental principle of the "dynamic state of body constituents."

In general, these experiments revealed that the deuterium concentration of the saturated fatty acids was about 50 percent of the deuterium in the body water. From this it was concluded that, in the synthesis of fat from carbohydrate in animals, half of the stably bound hydrogen atoms in the synthesized fatty acids came from the body water (Bernhard and Schoenheimer, 1940).

TABLE 1. TOTAL LIPIDS, FATTY ACIDS AND UNSAPONIFIABLE MATTER FROM MYCELIUM

Experiment No.	Total weight of mycel.	Total lipids of mycelia		Fatty acids		Unsaponifiable matter	
	g	g	%	g	%	mg	%
1	11.497	2.480	21.5	2.146	86.6	157	6.3
2	11.990	2.683	22.3	2.327	87.0	142	5.3
3	12.269	2.620	21.3	2.206	84.5	152	5.8
4	10.532	2.093	19.8	1.720	82.5	104	5.0
5	9.964	1.777	17.9	1.466	82.7	98	5.5

There are molds as well as certain yeasts which have the ability to produce fats in appreciable amounts and store them in their cells. This makes it possible to follow the synthesis of fats under simplified conditions. In contrast to the situation found with animals, we can begin with a fat-free medium. After a short time, for example with the formation of the mycelia, the lipid formation apparently comes to a standstill.

We have found that a mold, *Phycomyces Blakesleeanus*, grown in a glucose-containing medium, produces a mycelium which contains about 20 percent lipids. These contain 10 percent phosphatides and 5.3 percent unsaponifiable material. The latter consists mostly of ergosterol together with α- and β-carotene, and probably lycopene. The fatty acids contain 23.7 percent palmitic, 4.7 percent stearic, 1.7 percent behenic, 2.1 percent tetracosanoic, and 1.3 percent hexacosanoic acids. Among the unsaturated fatty acids we found 29.6 percent oleic and 25.8 percent linoleic acids, together with 3.4 percent γ-linolenic acid ($\Delta^{6:7,\ 9:10,\ 12:18}$-octadecatrienoic acid), which has previously been reported in nature only once. There was also obtained from the fatty acids 2.9 percent of a hitherto unknown singly-unsaturated fatty acid with 24 carbon atoms, which has the structure of a Δ17:18-tetracosenoic acid, and 4.9 percent of a hexacosenoic acid (Bernhard and Albrecht, 1948).

After the ability of this mold to synthesize appreciable amounts of a wide variety of lipids was demonstrated, it was felt that this was an organism suitable for the study of lipid synthesis in plants by following the uptake of hydrogen from the medium into fatty acids.

Dr. Albrecht and I cultivated the mold in a medium which contained 50 gm. glucose, 2 gm. L-asparagine, 1.5 gm. potassium dihydrogen phosphate, 0.5 gr. magnesium sulfate, 1 gr. yeast extract, and 40 γ thiamin per liter. Deuterium oxide was added to the doubly distilled water. As the heavy water was available only in limited quantities, we had to make several runs, recovering the water each time. The medium was used in 50 cc. portions in 300 cc. Erlenmeyer flasks. After sterilization we inoculated with ripe adult spores. In total, five experiments were performed, each with 40 cultures. The growth stopped after 10 days and the mycelia were collected, dried and weighed. They were ground with sand, warmed with methanol to split the lipoproteins and extracted thoroughly with methanol and ether. The lipids were saponified with alcoholic potassium hydroxide, the unsaponifiable material separated and the fatty acids isolated (see Table 1).

The sterols were obtained from the unsaponifiables by precipitation with digitonin. These and the fatty acids were analyzed for deuterium. The results are given in Table 2.

It appeared of interest to determine whether the deuterium concentrations were the same for all the individual fatty acids. Distinct differences might indicate different synthetic mechanisms. Due to the small yields of fatty acids, a separation of the individual components could be accomplished only by pooling the material obtained from all five experiments. The assumption was made that the five mixtures had the same fatty acid composition. This was probably true as the growth conditions were the same and the yields of mycelia and lipids did not differ appreciably.

The pooled total fatty acids (8.5 gm.) were separated through the lead salt into solid and liquid fractions. The latter weighed 4.395 gm. (54.9%)

and its deuterium content was 1.70 atom percent. This yielded on bromination the hexabromide of γ-linolenic acid, melting at 195° C., and the tetrabromide characteristic of cis-cis-linoleic acid. By crystallization at low temperatures the dibromide of oleic acid was obtained.

The solid acids (1.77 atom %) were methylated and fractionally distilled with the column described by Schoenheimer and Rittenberg (1937). Ten fractions were obtained, some of which were redistilled. The distillate was saponified and the acids recrystallized from suitable solvents. The separations of the tetracosanoic from the tetracosenoic acid, and of the hexacosanoic from the hexacosenoic acid were accomplished by means of the magnesium salts (Klenk, 1927).

The deuterium determinations of all fractions were carried out in duplicate. Some samples were too small for direct analysis and stearic acid was therefore added as carrier. The bromides were burned

TABLE 2. DEUTERIUM CONTENT OF CULTURE MEDIUM, FATTY ACIDS AND ERGOSTEROL

Experiment No.	Atom % deuterium in			Deuterium content in fatty acids and ergosterol relative to that of the medium†	
	Culture medium	Fatty acids	Ergosterol	Fatty acids	Ergosterol
1	4.61	2.90	2.59	63	56
2	3.51	2.16	—	62	—
3	2.75	1.64	1.51	60	55
4	1.61	0.99	—	62	—
5	1.41	0.91	—	64	—

† $\frac{\text{Atom \% deuterium in acids or ergosterol}}{\text{Atom \% deuterium in medium}} \times 100.$

together with silver oxide powder. The deuterium contents obtained are given in Table 3.

It was to be expected that the labile hydrogen atoms of the glucose would exchange partially with the deuterium of the medium. Our experiments showed that equilibrium was quickly attained, and the D-concentration of the glucose apparently did not change during the 10-day growth period (Table 4). However, formation of carbon-bound deuterium did not take place. Glucose isolated as the pentaacetate from the same samples was practically free of deuterium.

The D-values of the total fatty acids were about 62 percent of that of the medium water; the values for the different runs were, for biological experiments, in good agreement. It was our experience that the growth of the mold came to a standstill under our conditions after approximately ten days. It can be assumed that when this point is reached no changes in fat content of the mycelia occur; in any case no synthesis takes place.

TABLE 3. DEUTERIUM CONTENT OF THE FATTY ACIDS ISOLATED FROM THE LIPIDS PRODUCED BY *Phycomyces Blakesleeanus*

	Acid	Atom—% D
C_{16}	Palmitic	1.72
C_{18}	Stearic	1.79
	Oleic	1.69
	Linoleic	1.79
	γ-Linolenic	1.69
C_{22}	Behenic	1.73
C_{24}	n-Tetracosanoic	1.85
	n-Tetracosenoic	—
C_{26}	n-Hexacosanoic	1.80
	n-Hexacosenoic	1.75

It can be seen that the D-concentrations of the fatty acids, of which we isolated 10 different pure components, were not markedly different and varied within the limits of the error of measurement. It should be pointed out especially that the unsaturated fatty acids had D-contents similar to those of the saturated acids. Our experiments on lipid formation from carbohydrates in mice and rats (Bernhard and Schoenheimer, 1940; Bernhard, Steinhauser and Bullet, 1942), after enrichment of the body water with heavy water, had shown that the linoleic and linolenic acids isolated from the fat were always free of deuterium, from which it was concluded that these animals cannot synthesize these acids.

The D-values of ergosterol were 56 and 55; these are distinctly lower than those of the fatty acids. No details of the synthesis of this compound are known. The animal organisms may use acetic acid for the formation of cholesterol, according to Bloch and Rittenberg (1942).

It may be that fat synthesis from glucose begins with the formation of glycerol and pyruvic acid, and that the latter is decarboxylated to acetaldehyde. From this, by condensation and oxidation-reduction, aldol, crotonaldehyde, 2-hexenal, and finally capronaldehyde may be obtained. This may continue by further addition of acetaldehyde. Another possible route may consist in the formation of lactic acid, from which formic acid and acetaldehyde may be

TABLE 4. DEUTERIUM CONTENT OF GLUCOSE FROM THE CULTURE MEDIUM

Experiment No.	Atom % deuterium in		
	Water of culture medium	Glucose Before Development of the microorganisms	Glucose After Development of the microorganisms
1	4.61	1.26	1.25
2	3.51	1.22	—
3	2.75	1.53	—
4	1.61	0.74	0.79

produced. The latter could be condensed as above, and the formic acid might furnish the hydrogen for the reduction of the unsaturated aldehydes.

According to Günther and Bonhoeffer (1939), about 60 percent of the hydrogen atoms of the fatty acids formed in the course of these syntheses must in both cases be taken from the milieu and the ratio D/H (fat): D/H (water) must be about 0.6. We found 0.62, 0.61, 0.59, 0.61, and 0.64. Our experiments have therefore resulted in data which support the above theory that fat formation in plants goes through aldol condensations. The utilization of acetic acid for the synthesis of fatty acids by intact animals has been demonstrated with the acid of labeled acetate by Rittenberg and Bloch (1944).

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THE BIOLOGICAL SYNTHESIS OF LIPIDS

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The application of the tracer technique to the study of intermediary metabolism has led to the recognition that the aliphatic chains and hydroaromatic structures of lipids arise biologically by processes which employ small molecules as building stones. Acetic acid, whose role as a precursor has been studied most extensively, has proven to be a major source of carbon atoms for fatty acids and steroids in a variety of biological systems, *viz.*, bacteria, yeast and animal tissues. While the participation of two-carbon units in lipid synthesis is well established, little has been learned concerning the series of intermediate reactions by which the elementary units are successively linked to form aliphatic chains. It is believed that fatty acids might be synthesized by a reversal of the steps which lead to fatty acid degradation, namely, the formation of β-keto acids and their subsequent reduction to saturated acids, but direct experimental evidence in favor of this view is entirely lacking. The role of acetic acid in fatty acid synthesis is perhaps most clearly understood in those cases in which the end product of the reaction is a fatty acid of low molecular weight. In *Clostridium kluyveri* and *Cl. butylicum*, the short chain fatty acids, butyric and caproic acids accumulate as products of the dissimilation of acetate and ethanol. Fermentation of C^{14} acetate by these microorganisms yields C_4 and C_6 fatty acids with an isotope distribution which is clearly the result of repeated condensations of two carbon units as the only building stones (Wood, Brown and Werkmann, 1945; Barker, Kamen and Bornstein, 1946.) Experiments by White and Werkmann (1947) on the utilization of acetate for fat synthesis by yeast likewise demonstrate that the fatty acids synthesized by this organism can be quantitatively derived from acetic acid.

In the tissue lipids of animals, short chain fatty acids are present in negligible amounts only, and information on the mechanism of fat synthesis must therefore be derived from an analysis of the processes leading to the higher fatty acids which contain mainly 16 and 18 carbon atoms. In rats and mice which receive labeled acetic acid, isotope is incorporated into the fatty acids indicating that in animal tissues also acetic acid is a carbon source for fatty acids (Rittenberg and Bloch, 1945). By means of chemical degradation of the labeled products it can be shown that the isotopic carbon or hydrogen derived from acetate is uniformly distributed along the fatty acid chain and that therefore acetate can supply carbon atoms for the entire fatty acid chain. It may therefore be concluded that the mechanism of fatty acid synthesis by multiple condensation of two-carbon units is in principle the same for most biological systems. It is not known, however, whether in animal tissues acetic acid is quantitatively as important an intermediate in fatty acid synthesis as it appears to be in yeast and microorganisms. Synthesis of fat occurs in animals regardless of whether or not fat is contained in the diet. When fat is excluded from the diet, protein and carbohydrate are the evident sources from which fatty acids must be derived. One central problem in the study of the mechanism of fatty acids synthesis is therefore the nature of the intermediates which arise in the transformation of carbohydrate and protein to fat. It has been widely assumed that pyruvic acid is a key intermediate in fat formation and this view has been substantiated by experiments of Gurin, Delluva and Wilson (1947) with labeled lactate and by those of Anker (1948) with labeled pyruvate. Isotopic carbon from these compounds is readily incorporated into the tissues fatty acids of normal or phlorhizinized rats. Since both acetate and pyruvate have been established as sources of fatty acid carbon it may be asked whether these two precursors are transformed into a common intermediate which constitutes the primary building unit for the fatty acid chain, or whether the synthetic process involves a condensation of C_2 and C_3 units with subsequent decarboxylation to a product having an even number of carbon atoms. The suggestion has been made that pyruvate is metabolized by way of a two-carbon unit but little direct evidence exists in favor of the view that animals can readily convert pyruvate to the same intermediate which can also arise from acetate. The contrasting behavior of acetic and pyruvic acids in various metabolic reactions such as the acetylation of foreign amines, the synthesis of cholesterol (Bloch and Rittenberg, 1945) or of uric acid (Sonne, Buchanan and Delluva, 1946) speaks against the conversion of pyruvate into what may be called the metabolically active form of acetic acid. Rittenberg and Bloch (1945) have therefore suggested that pyruvate may be employed directly for fatty acid synthesis without passing through the acetic acid stage. This could be accomplished by the formation of acylpyruvic acids which would in turn be decarboxylated. The result would be a chain elongation equivalent to that caused by the addition of acetate. In view of the random distribution of labeled carbon in the fatty acids formed biologically from acetate it is mandatory to assume that pyruvate carbon or the carbon from any other precursor be also distributed uniformly in the fatty acid skeleton. This condition is fulfilled by a mechanism which visualizes an interchangeable use of

acetate and pyruvate for every two-carbon portion of the fatty acid, without requiring a conversion of pyruvate to acetate. Nor will it then be necessary to assume that C_2 units from acetate and pyruvate enter the final product in a fixed ratio under all conditions.

For the purpose of evaluating the relative contribution of acetate and pyruvate carbon it is not sufficient to determine the isotope content of the fatty acids after the feeding of labeled acetate and pyruvate respectively; it is also necessary to know the isotope concentration of the metabolites at the site of synthesis, *i.e.*, their isotope concentration in the metabolic "pool." The size of the acetic acid pool has been calculated (Bloch and Rittenberg, 1945) and it has been estimated that in mice a minimum of one-fourth of all fatty acid carbon atoms are supplied by acetic acid (Rittenberg and Bloch, 1945). The value for the size of the pyruvate pool is unknown and it is therefore not possible at present to determine the corresponding contribution of pyruvic acid to fatty acid synthesis.

TABLE 1. SYNTHESIS OF FATTY ACIDS IN RAT LIVER SLICES INCUBATED IN PHOSPHATE BUFFER CONTAINING D_2O
Time of incubation 3 hours

Additions 0.01 m	Atom % excess D* in total fatty acids
Acetate	.30
Fumarate	0.41
Oxaloacetate	0.66; 0.78
Pyruvate	0.96

* Calculated for 100 atom % D in the incubation medium.

In order to assess the roles of acetate and pyruvate in lipid synthesis we have carried out a number of *in vitro* experiments with surviving rat liver. We had previously observed that the formation of labeled cholesterol takes place readily in rat liver slices which are incubated in the presence of either D_2O or labeled acetate (Bloch, Borek and Rittenberg, 1946). Under the same conditions the uptake of isotope by the liver fatty acids is very small. A substantial increase of the rate of synthesis is obtained on addition of various intermediates of carbohydrate metabolism. Non-isotopic pyruvate, oxaloacetate or glucose, when added to the incubation medium which contains either D_2O or C^{14} acetate as markers, afford a several fold increase in the isotope concentration of the fatty acids (see Tables 1 and 2). When fatty acid synthesis is carried out in a medium of heavy water one obtains a measure of the overall synthetic rate since during total synthesis isotopic hydrogen must be fixed in the fatty acid chain irrespective of the nature of the precursor. On the other hand, the effects produced by various non-isotopic substrates on the incorporation of C^{14} from labeled acetate may be interpreted in various ways. Thus the increase in the uptake of both D and of acetate carbon caused by pyruvate is not necessarily the result of identical events. Pyruvate may either be a source of carbon or supply the energy required for the synthetic process. It is also possible that pyruvate enters into fatty acid synthesis indirectly by previous conversion to acetate but in this case one would expect pyruvate to dilute the isotope concentration of the labeled acetate and therefore diminish rather than increase the C^{14} content of the fatty acids. Of the various dicarboxylic acids which were tested only oxaloacetate showed stimulation and this is probably due to its decarboxylation to pyruvate. In contrast to oxaloacetate, malate fumarate and succinate markedly depressed the isotope incorporation from C^{14} acetate. No explanation can be given at present for the difference in behavior of oxaloacetate and the other C_4 dicarboxylic acids.

Some further information on the role of pyruvate in fatty acid synthesis has been obtained from experiments in which acetate labeled with C^{13} in the methyl group and pyruvate containing C^{14} at the carbonyl group (generously supplied by Dr. H. S. Anker) were simultaneously used and in which a third isotope was present in the medium in the form of D_2O. One may assume that under these conditions the marked substrates are not appreciably diluted by endogenous material, and that the incorporation of the C^{13} and C^{14} isotopes into the fatty acids provides therefore a measure of the utilization of

TABLE 2. SYNTHESIS OF FATTY ACIDS IN RAT LIVER SLICES FROM $CH_3C^{14}OONa$

Non-isotopic additions 0.01 m	Specific activity* of total fatty acids	
	Exp. 1	Exp. 2
None	0.14; 0.14	0.26
Pyruvate	—	.56
Glucose	0.33	—
Oxaloacetate	0.27; 0.53	0.40
Fumarate	0.08	0.14
Malate	—	0.08
Succinate	—	0.10

* In % of specific activity of acetate added.

acetate and pyruvate respectively. Experiments with intact animals have shown that in a newly synthesized saturated fatty acid molecule about three out of four hydrogen atoms are derived from the D_2O of the medium (Rittenberg and Schoenheimer, 1937). On this basis it is found that in liver slices in a three hour period, 0.5 percent of the saturated fatty acids were resynthesized when acetate was the only substrate and 0.9 to 1.1 percent when both acetate and pyruvate were present (see Table 3). The C^{13} values which measure the incorporation of ace-

tate carbon under these conditions likewise reflect the stimulation of fatty acid synthesis by pyruvate, although the increase in acetate utilization does not always parallel the increase in deuterium uptake. In the experiments in which both C^{13} acetate and C^{14} pyruvate were present, the C^{14} content of the fatty acids is low relative to their C^{13} concentrations demonstrating that under these conditions the fraction of fatty acid carbon atom derived from pyruvate was only one half to one fourth of that derived from acetate. If on the other hand, acetate is omitted from the reaction medium the incorporation of pyruvate carbon into the fatty acids is increased two to three fold and approaches the utilization values of acetate. It appears therefore that acetate limits the incorporation of pyruvate carbon into the fatty acids while on the other hand pyruvate stimulates the utilization of acetate carbon. The finding that the presence of acetate depresses the uptake of C^{14} from pyruvate might be ascribed to a conversion of pyruvate to acetate. In this case the acetic acid already present in the medium would dilute the C^{14} content of the acetate which arises from pyruvate. This explanation, however, fails to account for the fact that evidently the addition of pyruvate has no diluting effect on the isotope content of acetate but on the contrary increases the uptake of labeled carbon from acetate into the fatty acids. It is clear from these considerations that the role of pyruvate in fat synthesis is not merely that of a precursor for the two-carbon intermediates. Probably pyruvic acid as well as other intermediates of carbohydrate metabolism are needed to supply the large amounts of energy which are required for the conversion of acetic acid to the higher fatty acids. In addition, pyruvic acid may be an actual though not obligatory source of fatty acid carbon.

The simultaneous application of more than one isotopic tracer makes it possible to estimate not only the relative but also the total fraction of carbon atoms in a newly synthesized fatty acid molecule which is derived from acetate and pyruvate respectively. For this purpose the deuterium concentration of the saturated fatty acids synthesized in a medium of heavy water, multiplied by 1.33, is taken as the base value for total synthesis. The ratio of the isotope concentration in the fatty acids resulting from a specific precursor to this deuterium value, will then indicate the extent of utilization (see Table 3). In the case of C^{13} acetate the ratio $C^{13}/D \times 1.33$ is close to one when acetate is the only substrate, demonstrating that in this experiment practically all carbon atoms of the saturated fatty acids were derived from acetate alone. When acetate and pyruvate were present in the medium in equimolar concentrations, the corresponding ratio in two experiments was 0.6 and 1.0 respectively. On the other hand, as shown by the C^{14} values, pyruvate in the presence of acetate contributed only about one fourth of the carbon atoms in the fatty acid. The value for pyruvate utilization rose to 60 percent and 80 percent respectively when acetic acid was omitted from the incubation medium. It should be emphasized that the validity of these calculations rests on the assumption that three fourths of the hydrogen atoms of a saturated fatty acid are, under all conditions, derived from hydrogen which is in isotope equilibrium with the deuterium in the medium. However, the possibility cannot be ruled out that hydrogen employed for the reduction of keto groups is transferred directly from a non-isotopic hydrogen donor. In this case the deuterium content of the fatty acids would not be indicative of the overall rate of synthesis.

TABLE 3. ISOTOPE CONCENTRATIONS IN SATURATED FATTY ACIDS FORMED IN LIVER SLICES INCUBATED IN THE PRESENCE OF D_2O, $C^{13}H_3COONa$, AND $CH_3C^{14}OCOONa$

Additions 0.01 m	D*×1.33	C13*	C14†
C^{13} acetate	.57	0 54	—
C^{13} acetate+C^{14} pyruvate	1.06	0.60	0.28
C^{13} acetate+C^{14} pyruvate	—	0.68	0.25
C^{13} acetate+C^{14} pyruvate	0.91	0.91	0.23
C^{14} pyruvate	0.85	—	0.56
C^{14} pyruvate	—	—	0.69

* Atom % excess calculated for 100% C^{13} or D in labeled compound added.

† In % of average specific activity of α and β carbon atoms of pyruvate.

The data on the utilization of acetate and pyruvate respectively for fatty acid formation illustrate the flexibility of the synthetic process. Acetate appears to be the precursor which is utilized preferentially, and which can serve as the sole source of carbon atoms. Pyruvate is an alternative source which can be used interchangeably with the two-carbon units derived from acetic acid. It appears from the present experiments that the extent to which pyruvate contributes carbon for fatty acid synthesis is variable and inversely proportional to the availability of other precursors such as acetate. The experiments on fat formation which have been carried out by Anker (private communication) indicate that in intact animals, acetate rather than pyruvate is the major source of carbon atoms.

The fatty acids of rat tissue are composed of approximately one third saturated and two thirds unsaturated fatty acids. A number of years ago it was shown by Schoenheimer and Rittenberg (1936, 1937) that the saturated and monounsaturated fatty acids are interconvertible biologically by hydrogenation and dehydrogenation. The same authors have found that the unsaturated acids which are synthesized by mice in a heavy water medium contain considerably less deuterium than the saturated fatty acids (1937a). According to Bernhard and Bullet

(1943), the oleic acid synthesized by rats under similar conditions contained on an average only 40 percent as much deuterium as the saturated fatty acids, irrespective of the duration of the experiment. If oleic acid were exclusively formed by desaturation of stearic acid then it should ultimately attain the same isotope concentration as the C_{18} saturated acid. Since this was not the case, Bernhard and Bullet made the suggestion that a second independent mechanism may exist for the synthesis of monounsaturated fatty acids. It is conceivable that a portion of oleic acid arises by partial hydrogenation of linoleic or linolenic acids. These essential acids are not synthesized in animals tissues and therefore no

TABLE 4. INCORPORATION OF C^{14} FROM $CH_3C^{14}OONa$ INTO FATTY ACIDS IN RAT LIVER SLICES. Radioactivity of samples as counts/min. of $BaCO_3$ at infinite thickness

Non-isotopic additions	Fatty acids			Unsaturated
	A total	B saturated	B/A	
1) Fed rats				
None	53	63	1.2	—
Pyruvate	132	384	2.9	—
Pyruvate	148	393	2.7	—
Pyruvate	330	660	2.0	120
Fumarate	24.8	17.2	0.7	—
2) Starved rats				
Pyruvate	12.5	10.5	0.80	—
Pyruvate	15.8	11.6	0.73	—
Pyruvate	28.1	20.9	0.75	—

deuterium from labeled body fluids is introduced into their carbon skeletons. Oleic acid originating from this source would therefore contain only a small fraction of the amount of deuterium which would enter as the result of total synthesis from small units. This pathway from the essential fatty acids could, however, account only for a small portion of the newly formed oleic acid since the quantities of linoleic and linolenic acids in animal tissues are small compared to those of oleic acid.

Our experiments with liver slices contain evidence to strengthen the view of separate synthetic processes for saturated and monounsaturated fatty acids. As was found to be the case in intact animals (Rittenberg and Bloch, 1945), in liver slices also much larger amounts of acetate carbon are incorporated into the saturated than into the unsaturated fatty acids. Table 4 shows that in the presence of labeled acetate and non-isotopic pyruvate the ratio of the isotope concentrations in the saturated to total fatty acids lies somewhere between 2 and 3, and that therefore the saturated fatty acids must have a much higher isotope concentration. In one case the isotope concentration of the unsaturated fatty acids was determined and found to be only one fifth of that of the saturated fatty acids. The value for the monounsaturated acids must actually be somewhat higher since the fraction analyzed included all acids which form alcohol-soluble lead salts and therefore contained also the non-isotopic linoleic and linolenic acids. The difference of isotope concentrations in saturated and unsaturated acids is not constant but varies significantly depending upon experimental conditions. From the results which we have obtained so far it appears that the ratio has a large value whenever the overall rate of fatty acid synthesis is fast. On the other hand, in the absence of substrates which stimulate the synthetic process the ratio is only a little higher than one and becomes significantly smaller than unity in the presence of fumarate or in livers from starved animals. In slices prepared from rats which had been starved for 24 hours the rate of fatty acid synthesis is greatly reduced but evidently not proportionally for saturated and unsaturated acids. It can be estimated that under these circumstances the unsaturated acids are actually synthesized at twice the rate of the saturated acids, and probably not much slower than in livers of well fed animals. Thus, the rate of synthesis of the saturated fatty acids appears to vary much more markedly than that of the unsaturated acids. It is evident that under conditions which give rise to higher isotope concentrations in the unsaturated than in the saturated fatty acids, their synthesis must involve a process other than dehydrogenation of the corresponding saturated compounds.

We have reported (Bloch and Kramer, 1948) that insulin is capable of stimulating the utilization of acetate for fatty acid synthesis in liver slices. This action of insulin could be demonstrated when pyruvate was present in the incubation medium but not in the presence of acetate alone or in the presence of acetate plus glucose. We were therefore led to conclude that insulin might be involved in the utilization of pyruvate for fatty acid synthesis. Our original experiments were carried out with tissues taken from rats of a laboratory strain of unknown origin. More recently, we have used rats of the Sprague-Dawley strain and have not been able to reproduce regularly the stimulatory effect of insulin. It is not inconceivable that sensitivity to hormones varies in different strains of the same animal species, a possibility which is suggested by the findings of Krahl and Cori (1947), that rats of the Sprague-Dawley strain are less susceptible to alloxan, as measured by the rise of their blood sugar, than rats of other strains. From recent experiments we have obtained evidence that the effect of insulin on fatty acid synthesis can, under certain conditions, be demonstrated also in livers from the Sprague-Dawley rats. The rate of fatty acid synthesis by liver slices in a medium containing acetate and pyruvate falls off after two to three hours but was found to be maintained at its original level for three

more hours when insulin was present. Further experiments will be necessary to determine whether or not the effect of insulin on fatty acid synthesis is of general significance.

One of the difficulties attending the study of fatty acid synthesis *in vitro* is the great sensitivity of the enzyme systems involved. Although in parallel experiments of one series with aliquots of pooled slices the variations which occur are only slight, the metabolic activity of surviving liver with respect to fatty acid synthesis varies greatly from day to day. The level of activity in one series of experiments compared to another may differ by as much as a factor of 5 in spite of the fact that experimental conditions are the same in all respects. The highest isotope concentration which we have found in the saturated fatty acids corresponds to a replacement of 1.3 percent in a three hour period. This value is somewhat less than half of that expected from the replacement values of liver fatty acids in intact rats (Stetten and Grail, 1943). It is also of interest to calculate the portion of the acetic acid which is converted to fatty acid. This value likewise varies considerably from experiment to experiment and depends not only on the rate of synthesis but also on the quantity of acetate present in the incubation medium. Since no significant change occurs in the isotope concentration of the fatty acids when the total quantity of acetate is varied from 0.05 molar to 0.5 molar, the fraction utilized will be larger when the amounts of acetate in the medium are small. At the lowest level tested (0.05 molar) it was found that maximally about 6 percent of the added acetic is converted to fatty acid.

Synthesis of Cholesterol

Our earlier investigations on cholesterol synthesis had indicated that the mechanisms for the synthesis of cholesterol and fatty acids must differ in some essential aspects although acetate is the principal building unit in both processes. Specifically, it has been contended that the higher fatty acids cannot be intermediates in the conversion of acetic acid to steroids, since in feeding experiments with deuterio acetate newly synthesized cholesterol contained several times the isotope concentration of the fatty acids (Bloch and Rittenberg, 1942). In liver slices also, labeled cholesterol is readily formed and in some instances more than 20 times as much acetate carbon is found to be incorporated into the cholesterol than into the fatty acids isolated from the same experiment. The pronounced differences of the two synthetic processes are also evident from data of the present experiments. The synthesis of cholesterol appears to proceed optimally when acetate is the only substrate in the incubation medium. Addition of pyruvate which stimulates fatty acid formation, has an opposite effect on the incorporation of acetate carbon into cholesterol. From the data in Table 5 it can be seen that with increasing uptake of C^{14} by the fatty acids under the influence of pyruvate, the isotope concentration in cholesterol declines. As has been pointed out above, a conversion of pyruvate to acetate and a consequent diluting effect on the isotope concentration of acetic acid does not occur significantly under our experimental conditions. Therefore the lowering of the isotope concentration in cholesterol caused by pyruvate most likely reflects an inhibition of overall synthesis.

Table 5. Effect of Pyruvate on the Incorporation of $CH_3C^{14}OONa$ into Fatty Acids and Cholesterol in Rat Liver Slices

Molarity of pyruvate	Specific activity* of	
	Total fatty acids	Cholesterol
0.003	0.036	0.50
0.01	0.072	0.41
0.02	0.11	0.21

* In % of specific activity of acetate added.

Table 6. Incorporation of Isotope into Liver Cholesterol *in vitro* from $C^{13}H_3COONa$ and $CH_3C^{14}OCOONa$

Additions 0.01 m	C^{13}*	C^{14}†
C^{13} acetate	1.37	—
C^{13} acetate+C^{14} pyruvate	0.87	0.03
C^{13} acetate+C^{14} pyruvate	.79	.03
C^{14} pyruvate	—	0.08

* Atom % excess calculated for 100% C^{13} in acetate added.
† In % of average specific activity of α and β carbon atoms of pyruvate.

The role of pyruvate as a source of carbon for cholesterol synthesis has been tested by isolation of the steroids from the experiments in which both C^{13} acetate and C^{14} pyruvate were used as substrates. As in previous experiments the incorporation of isotope into cholesterol was highest when acetate alone was present and reduced to about half by the addition of an equimolar quantity of pyruvate. From the data in Table 6 it is clear that pyruvate does not contribute significantly as a carbon source to cholesterol formation. The number of pyruvate carbons employed for cholesterol synthesis could not have been more than 1/30 of the number of carbon atoms derived from acetic acid. It is noteworthy that the ratio of pyruvate carbon to acetate carbon in cholesterol is only about 1/10 of the corresponding ratio in the fatty acids which were simultaneously formed. Evidently pyruvate can provide two-carbon units which are employed for fatty acid formation but this same two-carbon unit cannot be interchanged for acetate in cholesterol synthesis.

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Discussion

VILLEE: Experiments in our laboratory in which rat diaphragm muscle was cultured *in vitro* in a phosphate buffer have given results similar in most respects to those of Dr. Bloch using liver slices. We have found that C^{14} alpha labeled pyruvate was incorporated into the total lipid fraction at a greater rate than was C^{14} carboxyl labeled acetate and that the addition of non-labeled pyruvate to the acetate did not increase significantly the acetate incorporation. We have found a considerable effect of insulin in increasing the rate of incorporation of pyruvate into lipids. Without insulin 1.1 percent of the pyruvate uptake is incorporated into lipid; when insulin is added the amount incorporated is increased to 1.8 percent of the pyruvate uptake. Insulin does not increase the amount of pyruvate oxidized to CO_2 nor the incorporation of pyruvate into proteins. An increase in the amount of lipids present occurs during the two hour incubation period and this increase is greater with than without added insulin.

THE USE OF ISOTOPES IN AN INTEGRAL EQUATION DESCRIPTION OF METABOLIZING SYSTEMS

HERMAN BRANSON

The complex structure and multiple reactions occurring in most metabolizing systems make it impossible at present to find a mathematical treatment adequate for a description of the microscopic phenomena. By ignoring some of the details of such systems and giving our attention to quantities which are directly measurable, a mathematical description, which is eminently satisfactory for many purposes, is possible through integral equations. The literature of the equations encountered is extensive. Thus if the procedure is acceptable for the biological problem, the method will open a broad range of applicable mathematics to the biophysicist for discussing metabolizing systems.

Integral equations and integro-differential equations have often been suggested as being especially suited for the handling of biological systems which depend not only upon the immediately preceding states but upon all their previous states, for these equations are successful in describing such physical systems (*e.g.*, hysteresis phenomena). It is clear, however, that in order for an integral equation description to be preferred to a differential equation approach, the description should possess something more than logical superiority. The usual differential equation description of such complicated systems necessitates special assumptions about the mechanism of the reactions. The assumptions are often *ad hoc* and have a high pertinacity, so much so that the literature is studded with mechanisms which are hardly warranted by the data. The integral equation approach will improve, in large measure, this situation. But the treatment must also be more suggestive and yield mathematical functions more adequate for the problem at hand.

This paper essays a mathematical treatment of metabolizing systems which describes some important characteristics of such systems in terms of a rate function and a metabolizing function in an integral equation. The resulting equations are applied to several problems of biological and chemical interest. The equations are solved for functions derived from several sets of available data. Experimental procedures for determining the functions are discussed. The integral equations are shown to be valuable in problems employing isotopic tracers. Evidence is presented to support the view that this formulation may become a convenient means of correlating some of the work now being done with tracer molecules in biological systems. The procedure seems especially valuable in the application of "double-tracer" molecules, for it suggests a simultaneous determination of the rate function and metabolizing function of a metabolite by measurements of one substance in a single system.

The Integral Equation

A metabolizing system may be represented as an irregularly shaped region of complex structure where a substance or material is being produced,

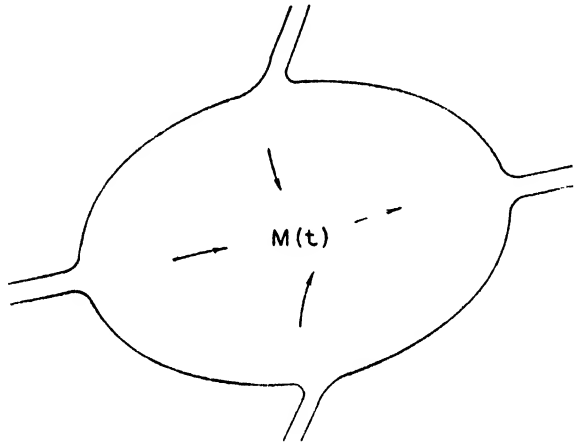

FIG. 1. Schematic representation of a metabolizing system. $M(t)$ is the amount of the metabolite present at time, t. $R(t)$ is the rate function and $F(t)$ is the metabolizing function of the system.

consumed, transported, modified, or stored. The system may be performing all or several of these functions simultaneously. The system may or may not have a definite boundary. For convenience the system may be thought of as in Figure 1 although the number of openings or points of diffusion through the walls does not affect our treatment. The following functions may be defined for this system:

$M(0)$ = amount of substance present initially, at $t=0$.

$M(t)$ = amount of the substance present at time t.

$R(t)$ = the rate at which the substance is accumulating in the system or disappearing from the system.

$F(t)$ = the "metabolizing function" to be defined presently.

In this system, the experimenter is concerned about some substance originally present in amount, $M(0)$. This substance is accumulating at a rate $R(\theta)$ so that the additional amount appearing at the time interval $\Delta\theta$ is $R(\theta)\Delta\theta$. This additional amount has the same fate as the amount originally present; it is metabolized. To account for this phenomenon a "metabolizing" function is introduced, which is defined as the function which will multiply the original amount, $M(0)$, to yield what remains of it at time, t. Thus, the amount remaining from the original amount at time, t, is $M(0)F(t)$; of the amount entering in the interval θ to $\theta+\Delta\theta$, there remains at time, t, $F(t-\theta)R(\theta)\Delta\theta$. We may divide the period 0 to t into the intervals, $\Delta\theta$ to $\Delta\theta_n$. Then the total amount present at time, t, from the original amount and the amount accumulating is

$$M(t) = M(0)F(t) + \sum_i F(t - \theta_i)R(\theta_i)\Delta\theta_i.$$

In the limit as the intervals approach zero, the summation becomes an integral so that the basic equation is

$$M(t) = M(0)F(t) + \int_0^t F(t - \theta)R(\theta)d\theta.$$

In the majority of tracer experiments, an experimenter knows $M(0)$ and determines $M(t)$. If equation (1) were used in correlating the data in such an experiment, we would have to know either $F(t)$ or $R(t)$ from another experiment. Equation (1) would be a Volterra integral equation of the second kind in $F(t)$ if we know $R(t)$ or a Volterra integral equation of the first kind in $R(t)$ if we know $F(t)$. Finally if $M(0)$, R, and F were known, the equation could be integrated either directly or numerically to find $M(t)$.

Applications to Some Chemical and Biological Systems

A large number of chemical and biological systems are characterized by the fact that the rate of reaction of the substance of interest is directly proportional to the quantity of the reacting substance present. This type of reaction is designated in chemical kinetics as a first order reaction. As a numerical example, the decomposition of dibromosuccinic acid in hot water investigated by Van't Hoff and treated by Hitchcock and Robinson (1936) may be considered.

All first order reactions are very easily treated by equation (1) with the following designations:

$$M(0) = \text{a known consonant}$$

$$F(t) = F(0) = 1.$$

$$R(\theta) = -kM(\theta).$$

Equation (1) becomes

$$M(t) = M(0) - K\int_0^t M(\theta)d\theta. \qquad (2)$$

Equation (2) is of the type

$$\phi(x) = f(x) + \lambda\int K(x, y)\phi(y)dy.$$

This Volterra equation of the second kind is solved by the Liouville-Neumann series (Margenau and Murphy, 1943).

$$\phi(x) = \sum_{n=0}^{\infty} \lambda^n\phi_n(x)$$

provided

$$\phi_0(x) = f(x);$$

$$\phi_1(x) = \int K(x, y)\phi_0(y)dy, \cdots, \phi_n(x)$$

$$= \int K(x, n)\phi_{n-1}(y)dy.$$

In the example,

$$K(x,y) = 1, \lambda = -K, \text{ and } \phi_0(x) = M(0).$$

Introducing these expressions and integrating, the solution becomes

$$M(t) = M(0)\sum_{n=0}^{\infty} \frac{(-Kt)^n}{n!} = M(0)e^{-Kt}. \qquad (3)$$

Equation (3) is the usual exponential expression which is more easily obtained in this instance by stating the relationship in differential equation form and integrating. With $k=0.030$, the equation is an adequate description of the decomposition of dibromosuccinic acid in the Van't Hoff experiment.

First order reactions in certain systems, however, seem possible of treatment in much more elegant manner through equation (1) than through differential equations. As an example, a system in which the reaction is of the first order and at the same time the material is flowing out constantly such that the "metabolizing" function is of the form $F(t)=(1-ct)$ may be considered. Using the designations as in equation (2), equation (1) becomes

$$M(t) = M(0)(1 - ct) - K\int_0^t M(\theta)[1 - c(t - \theta)]d\theta. \qquad (4)$$

This integral equation may be solved by the Liouville-Neumann series as used in equation (2);

however, it is desirable to introduce the powerful mathematical technique of the Laplace transform which is eminently suited for treating many of the problems described through equation (1).

The integral on the right of equation (1) is a special type referred to as "Faltung" integral (literally: a folding integral) by Doetsch (1943). Equation (1) may be written $M(t)=M(0)F(t)+F^*R$, where F^*R is the abbreviation for the integral. If the equation is multiplied through by $\exp(-st)$ and integrated from 0 to ∞, it becomes

$$\int_0^\infty M(t)e^{-st}dt = M(0)\int_0^\infty F(t)e^{-st}dt + \int_0^\infty \int_0^t R(\theta)F(t-\theta)e^{-st}dtd\theta. \quad (5)$$

The conditions on the functions in order that these integrations are permissible are discussed by Doetsch (1943). It is assumed that the integrations are allowed. By definition the integral on the left of equation (5) represents the Laplace transform of $M(t)$ which is called $m(s)$; the first integral on the right is the transform of $F(t)$ multiplied by $M(0)$, that is $M(0)f(s)$. The double integral on the right is the transform of the "Faltung" integral. Doetsch (1943) shows that the transform of F^*R is equal to the product of the individual transforms, or

$$L\{F^*R\} = L(F)\cdot L(R) = f(s)r(s).$$

Hence equation (5) may be written as

$$m(s) = M(0)f(s) + r(s)f(s) \quad (6)$$

where the lower case letters represent the transforms of the corresponding functions in capitals. An alternate statement is that equation (1) transforms into equation (6). If $f(s)$ is sought, equation (6) becomes

$$f(s) = \frac{m(s)}{M(0)+r(s)}. \quad (7)$$

It may happen that the particular form on the right side of equation (7) is one of the many Laplace transforms which have been tabulated or that it may be expressed in standard form through algebraic manipulation. If either circumstance exists, the inverse transform, $F(t)$, may be read directly from tables given, for example, in Doetsch (1943), Carslaw and Jaeger (1941), Churchill (1944), or MacLachlan (1939). If the right side is not a standard form, integration in the complex plane is required. The inverse transform is

$$F(t) = \frac{1}{2\pi i}\int_0 e^{st}f(s)ds.$$

Details of the integration may be found in the texts mentioned above.

Applying the Laplace transform technique to equation (4) transforms it into

$$m(s) = M(0)\int_0^\infty e^{-st}(1-ct)dt - K\int_0^t\int_0^\infty M(\theta)[1-c(t-\theta)]e^{-st}d\theta dt.$$

These integrations are easily performed or the transforms may be read directly from a table:

$$m(s) = \frac{M(0)}{s} - \frac{M(0)}{s^2} - \left(\frac{K}{s} - \frac{CK}{s}\right)m(s).$$

This equation may be written in terms of $m(s)$ with the right side in a standard form,

$$m(s) = M(0)\left[\frac{A}{s+\alpha} + \frac{B}{s-\beta}\right]$$

where

$$\alpha = \tfrac{1}{2}(K \pm \sqrt{K^2+4Kc}), \qquad A = \frac{\alpha+c}{2\alpha-K},$$

$$\beta = \tfrac{1}{2}(-K \pm \sqrt{K^2+4Kc}), \quad B = \frac{\alpha-K-c}{2\alpha-K}.$$

Consulting Doetsch's (1943) table again the inverse transforms are found,

$$M(t) = M(0)[Ae^{-\alpha t} + Be^{\beta t}]. \quad (8)$$

Equation (8) describes the behavior of a system in which a first order reaction is taking place and, at the same time, the reacting material is being transported away at a constant rate. It is evident, too, that equation (8) reduces to equation (3) when $c=0$.

In vitro experiments with tissue slices bathed continuously in a solution free of the metabolite in question may be described by equation (4).

A large number of biological systems under normal conditions are characterized by the fact that the amounts of certain metabolites remain constant. In terms of equation (1) the equation suitable for each such metabolite is

$$M[1-F(t)] = \int_0^t R(\theta)F(t-\theta)d\theta \quad (9)$$

where M is the constant amount present.

The procedure from this point is determined by what additional information is available. For example, if it may be known or suspected that the rate at which material enters the system is constant, the problem becomes one of determining the F-function. The resulting equation is easily shown to have the solution $F(t)=\exp(-R/M)t$. Thus a system in which the amount is constant is char-

acterized by an exponentially decreasing metabolizing function. The converse of this problem is also of biological interest. It is interesting to note that a metabolizing system with a constant amount and a constant F-function is impossible, for assuming that $R(t)$ is a well-behaved function, this system would require

$$\int_0^t R(\theta)d\theta = \text{constant}$$

which is not possible.

The rate of change of the amount of the metabolite may be found from equation (1) by differentiating,

$$\frac{dM}{dt} = M(0)\frac{dF}{dt} + \int_0^t R(\theta)\frac{\delta F(t-\theta)}{\delta t}d\theta + R(t)F(0). \tag{10}$$

This equation is of additional interest, for it may be used as the integral equation of the second kind for determining $R(t)$.

Tracer Experiments for the Determination of the Rate Function and the Metabolizing Function

In the typical experiment on metabolism, the experimenter is interested in the fate of some metabolite which we shall designate as $M(t)$. If the experiments are on mature, normal animals in nutritional equilibrium, it is often found that the amount of the metabolite varies little if at all. The introduction of a tagged quantity of a metabolite, $M(t)$, reveals that the constancy is only apparent. The equilibrium is dynamic, as has been so ably demonstrated by Schoenheimer and collaborators (Schoenheimer, 1946) with the stable heavy isotopes of hydrogen (H^2) and nitrogen (N^{15}). Hence when the metabolite is assayed, the results are on the flat portion of a curve whose origin cannot be determined by examining $M(t)$ at the time of our experiment. Clearly equation (9) is applicable in this situation with R and F to be determined. Another independent relation for determining one of them is necessary.

The tracer atom technique gives the required condition, for the experimenter need only inject a sample of tagged metabolite, $M^*(0)$, and follow its course in the same system or systems sufficiently similar. These data will give

$$M^*(t) = M^*(0)F(t). \tag{11}$$

Since no additional tagged metabolite can enter, $R^*=0$. Thus, by means of equations (9) and (11), we can determine the functions R and F.

The preceding formulation of equation (11) assumes that although the rate at which the tagged substance enters is different from that of the normal, the metabolizing functions are identical for both. If that were not true, then the system would be discriminating between the tagged and untagged molecules of otherwise identical constitution. Within the experimental error of this work, there is convincing evidence that the biological system cannot distinguish the small differences in mass between the chemically similar units. There may exist slight differences in rates of diffusion and other physical phenomena.

If the concern is for a metabolite or substance which is not in equilibrium, the procedure is the same except that, then, $M(t)$ must be determined and equations (1) and (11) used for R and F.

Treatment of Some Data from Tracer Experiments

As examples some experiments using radioactively tagged molecules of Hamilton and Soley (1940) and experiments and theoretical developments by Zilversmit and collaborators (1943) will be considered, and mention will be made of some experimental results of Schoenheimer and his collaborators (Schoenheimer, 1946).

Hamilton and Soley (1940) fed radioactive iodine (I^{131}) to a group of normal human subjects and observed by means of an external Geiger-Mueller counter the emanations from the thyroid. From the many types of empirical curves which may be fitted to their data, the writer selects

$$M(t) = (C)1 - e^{-\alpha t})e^{-\beta t}$$

with $C=0.035\ M^*(0)$, where $M^*(0)$ is the amount of tagged iodine fed. This choice of $M(t)$ describes the observed behavior in a satisfactory manner and the expression is sufficiently tractable mathematically for straightforward integration in equation (1). From their data it is seen that the maximum of the curve is reached in about one day, hence $\alpha \sim 4.5\ \text{days}^{-1}$. After 30 days, over 80 percent of the radioactive iodine remained in the thyroid, hence $\beta \sim 0.006\ \text{day}^{-1}$. Inasmuch as there was no radioactive iodine originally present, equation (1) now becomes:

$$C(1 - e^{-\alpha t})e^{-\beta t} = \int_0 R(\theta)F(t-\theta)d\theta.$$

Although an equation of the type of equation (11) cannot be written on the basis of the data, the fact that the radioactive iodine slowly leaves the thyroid limits the possibilities for $F(t)$. Upon integration and by use of this qualitative information on $F(t)$, suitable functions are found to be

$$\begin{aligned} R(t) &= \alpha e^{-(\alpha+\beta)t}, \\ F(t) &= e^{-\beta t}. \end{aligned} \tag{12}$$

Thus it may be concluded on the basis of our formulation that the metabolizing function describing the history of iodine in the thyroid is a

slowly decreasing function of time, while the rate function decreases rapidly with time. Although the parameters α and β are empirical and are not related to physiological processes, nevertheless, their values may be important clues in detecting malfunctioning of the thyroid.

The example given by Zilversmit and his collaborators (Zilversmit, Enteman and Fishler, 1943; Zilversmit, Enteman, Fishler and Chaikoff, 1943) is an instance of the general problem of the conversion of A into B where A is called the precursor of B. A may be produced by complex reactions and B may be lost through others. We shall assume only that for each unit of A which disappears a unit of B appears. If A and B are tagged substances, none of either is present initially, hence

$$A^*(t) = \int_0^t R(\theta)F(t-\theta)d\theta,$$

$$B^*(t) = \int_0^t R_1(\theta)F_1(t-\theta)d\theta.$$

If the assumption is made that the transformation of A into B is an irreversible, first order reaction, chemical kinetics states $R_1(\theta)=CA^*(\theta)$, then

$$B^*(t)=C\int_0^t\left[\int_0^\theta R(\phi)F(\theta-\phi)d\phi\right]F_1(t-\theta)d\theta.$$

The resulting expression for $B^*(t)$ is complicated, as one would expect, in the absence of additional simplifying assumptions. There are three functions to be determined, R, F and F_1. An additional relation is needed in order to determine the functions uniquely. One approach would be to follow the behavior of A^* and B^* in a similar system where it is injected. For such a system $A^*(t)=A^*(0)F(t)$.

The systems treated by Zilversmit and collaborators (Zilversmit, Enteman and Fishler, 1943; Zilversmit, Enteman, Fishler and Chaikoff, 1943) are simpler. Their general system ($A\rightarrow B$) is described in the integral equation formulation by the equations

$$A^*(t) = \int_0^t R(\theta)F(t-\theta)d\theta,$$

$$B(0) = B(0)F_1(t) + \int_0^t R_1(\theta)F_1(t-\theta)d\theta,$$

$$B^*(t) = C\int_0^t A^*(\theta)F_1(t-\theta)d\theta. \qquad (13)$$

These state that the amount of B present is constant, and $R=CA$ which is constant if the amount of A is constant. Thus from following the courses of A^*, B^*, and determining B in one system and either A in the same system, or A^*, or B^*, in a similar system, we shall have enough relations to determine the R's and F's. If the experimenter is interested only in the R and F associated with B, only $A^*(t)$, $B^*(t)$, and $B(0)$ need be known.

Their experiments on the turnover rate of phospholipids in the plasma of the dog with radioactive phosphorus follow the conditions for the system described by equation (9) for the ordinary phosphorus and $B^*(t)=B^*(0)F(t)$ for the radioactive phosphorus. Since R is constant, $F(t)=\exp(-R/M)t$.

The curves describing the atom percent deuterium uptake of cholesterol in mice (Schoenheimer, 1946), as well as other experimental results with stable isotopes (Schoenheimer, 1946), are expressed with acceptable accuracy by $M(t)=C(1-e^{-\alpha t})e^{-\beta t}$, with $\alpha\gg\beta$ which gives expressions similar to equation (13) for the metabolizing function and the rate.

An interesting example of the application of equation (1) is its use in a case formulated by Stetten and Boxer (1944) who state: "It may be shown that if the total quantity of any body constituent remains constant, if the amount replaced per unit time is constant, and if the synthetic process involves the uptake of isotope from a reservoir of constant concentration, the equation $\log_n(i_{max}/i_{max}-i)=kt$ represents the relationship between the isotope concentration of that body constituent and time." For this system the equations are

$$M(0) = M(0)F(t) + \int_0^t F(t-\theta)R(\theta)d\theta$$

and

$$M^*(t) = \int_0^t F(t-\theta)R^*(\theta)d\theta.$$

The conditions give $R(\theta)=C$ and $R^*(\theta)=C^*$. Inserting these values and solving by the Laplace transform result in

$$M^* = \frac{C^*}{C}M(1-e^{-Kt}) \quad \text{where} \quad K=C/M(0).$$

In the notation of Stetten and Boxer (1944) this equation is $i=i_{max}(1-e^{-kt})$ which may be transposed into the desired form.

Comparison of Integral Equation and Differential Equation Treatments of a Tracer Experiment

The work of Gellhorn, Merrell, and Rankin (1944) on transcapillary exchange of sodium in normal and shocked dogs investigated with Na^{24} is an excellent treatment of tracer data by the usual differential equation formulation. In this series of experiments, $Na^{24}Cl$ in 0.1 to 0.3 millicurie doses was given to dogs. Samples of blood were removed by arteriopuncture. The plasma

concentration of Na^{24} was plotted as a function of time. An equation was fitted to these data by the method of least squares. For normal dogs, they found

$$C_p - 1000 = 2117e^{-1.040t} + 933e^{-0.095t}. \quad (14)$$

In the mathematical discussion and presentation of their results, Gellhorn, Merrell and Rankin (1944) divided the body into three regions: the plasma, a region of fast exchange, and a region of slow exchange. They were able to establish a set of

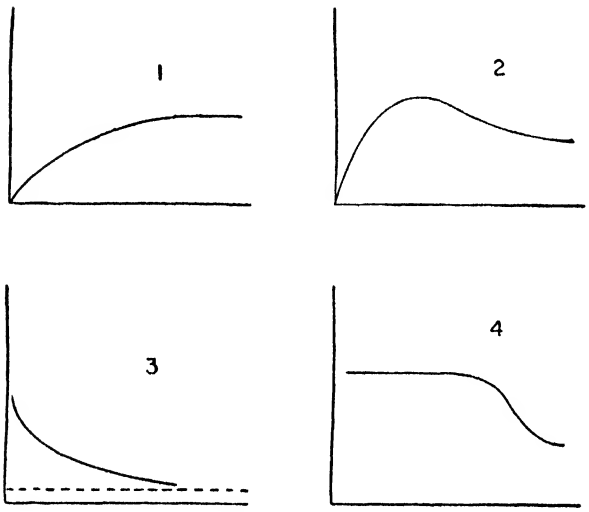

FIG. 2. Some curves of $M(t)$ as a function of time which occur frequently. In each graph, $M(t)$ is plotted along the vertical and time along the horizontal. In (1), $M(t)=at/(1+bt)$ or $c(1-e^{-\alpha t})$. In (2), $M(t)=c(1-e^{-\alpha t})e^{-\beta t}$ with $\alpha \gg \beta$. In (3), $M(t)=a+be^{-\alpha t}+ce^{-\beta t}$. In (4), $M(t)$ is constant for $0 \leqq t < r$ and $M(t)=a+be^{-\alpha t}+e^{-\beta t}$ for $t \geqq r$.

three first-order differential equations which on integrations gave an equation of form (14).

These data may be discussed by the integral equation formulation without recourse to any special assumptions although the resulting equations may be interpreted in terms of a model.

In applying the integral equation formulation, $M(t)$ is defined as the concentration of Na^{24} atoms per cc. Then from the experimental data, equation (1) becomes

$$M(t) = a + be^{-\alpha t} + ce^{-\beta t}$$
$$= M(0)F(t) + \int_0^t R(\theta)F(t-\theta)d\theta.$$

Since all of the Na^{24} present in the plasma is counted irrespective of the chemical combination. $F(t)$ may be set equal to one. Then

$$a + be^{-\alpha t} + ce^{-\beta t} = M(0) + \int_0^t R(\theta)d\theta$$

which is easily integrated to yield

$$R(t) = -\alpha e^{-\alpha t} - \beta ce^{-\beta t} \text{ since } M(0) = a + b + c.$$

Inasmuch as the total number of radioactive atoms present at time t is $M(t)$, the fractional transfer of Na^{24} from the plasma is

$$\frac{\alpha be^{-\alpha t} + \beta ce^{-\beta t}}{M(t)}.$$

Since the system does not distinguish between Na^{23} and Na^{24}, the proportion of sodium transferred at $t=0$ is

$$\frac{\alpha b + \beta c}{a + b + c} = 0.57$$

from the experimental values in equation (14). This and other results in the Gellhorn, Merrell, and Rankin (1944) paper seem to be derivable from the integral equation treatment more directly and without special assumptions.

Procedure in Applying the Integral Equation Treatment

In applying this integral equation to most systems where $M(t)$ is determined experimentally by the use of tracers or otherwise, the following procedure is recommended.

1. Plot the data as a simple graph of $M(t)$ *vs.* t.
2. From the shape of the curve, try special graphs (*e.g.* semilogarithmic).
3. Fit a continuous function to the graph by the method of least squares or the method of averages. The method of averages is simpler and usually satisfactory. In fitting curves, the data usually do not warrant the application of criteria of suitability.
4. Use the continuous function as $M(t)$ in the integral equation.
5. From another system, find $F(t)$ in the same manner or predict the form of $F(t)$ from the information on the single system.

Figure 2 shows some curves which occur frequently in metabolism experiments and suggested forms to try for $M(t)$. In following this procedure, the texts by Whittaker and Robinson (1924), Running (1917), and especially Worthing and Geffner (1943) are most helpful.

Tracer Experiments with Doubly-Tagged Molecules

"Doubly-tagged molecules" designate a substance part of whose molecules are tagged by one isotope and part by another; *e.g.* methionine, with some molecules having S^{34} and some S^{35}, or betaine, with some molecules having N^{15} and some C^{13}. The use of such doubly tagged molecules is not yet extensive in tracer experiments. But it is expected that with the greater availability of mass

spectrometers and the refinement of techniques for preparing or synthesizing the molecules, doubly tagged molecules will be most valuable in such experiments. The integral equation formulation shows how they will be particularly useful in characterizing a metabolizing system.

In the consideration of the reaction $(A \rightarrow B)$ in equations (13), it was seen that $A^*(t)$, $B^*(t)$, and $B(0)$ must be known in order to determine R and F for the system. In most complex biological systems even the reactions postulated by equations (13) would be in doubt, for they require proof that B is the exclusive product of A. But under the dynamic conditions existing in biological systems, the experimenter can expect, in addition to $(A \rightarrow B)$, side reactions and complex chains, some of which may also lead eventually from A to B.

One procedure for eliminating the dependence upon measurements of $A^*(t)$ would be to introduce the tagged substance suspected as being the precursor of $B^*(t)$ in one system or series of animals and the tagged substance, $B(t)$, in a similar system. From the first system we have:

$$B^*(t) = \int_0^t R(\theta)F(t-\theta)d\theta, \qquad (15)$$

and from the second system.

$$\overline{B}(t) = \overline{B}(0)F(t) \qquad (16)$$

which are sufficient to determine $F(t)$ and $R(t)$ without dependence upon precursors of B.

Although of value, the preceding technique is probably as vulnerable as the first, since the measurements are made upon different systems. The experimenter would have greater confidence in his rate determinations if they were based solely upon measurements of the substance under study and limited to simultaneous measurements in a single system.

These desired conditions may be obtained by the use of a doubly-tagged substance in a single system. We may introduce the suspected tagged precursor of B and follow experimentally the level of $B^*(t)$. At the same time, we may inject some $\overline{B}$, that is, a small amount of the chemically similar substance but tagged by the use of a different radioactive isotope or by a rare stable isotope. Thus equations (15) and (16) permit the simultaneous determination of $R(t)$ and $F(t)$ in a single system by measurements of B alone.

The number of possible combinations of isotopes available depends in large measure upon the problem and the skill of the experimenter. Under all circumstances, radioactive and stable pairs such as (H^2, H^3), (C^{13}, C^{14}), (S^{34}, S^{35}) and the radioative pair (Fe^{55}, Fe^{59}) can be used for these elements. No appropriate radioactive member exists for oxygen or nitrogen; however, in many experiments they can be coupled with hydrogen, carbon, or sulfur.

Experiments with such doubly-tagged molecules are being planned in our laboratory. Unfortunately our mass spectrometer, which is having leak trouble, and our work with P^{32} have not permitted us to present any data in support of this integral equation formulation. It seems clear, however, that the procedure will give a $R(t)$ and $F(t)$ as defined by the integral equations.

The Uniqueness of $R(t)$ and $F(t)$

In the discussion of the data of Hamilton and Soley (1940) and that of Gellhorn, Merrell and Rankin (1944) a certain arbitrariness was revealed in the choice of $F(t)$ and $R(t)$. Clearly in the Hamilton and Soley (1940) experiment, $F(t)$ could have been taken equal to one and $R(t)$ found so as to satisfy the equation for the $M(t)$ fitted to the curve. This arbitrariness would not have existed, however, had we performed two experiments as discussed in connection with equations (2) and (3). But even in this instance, the quantitative information suggests that $F(t)$ should not be taken as constant. Hamilton and Soley (1940) found that there was a rapid uptake and slow release. This information implies that a given amount of iodine initially in the thyroid would leave gradually. According to our formulation this quantity of iodine should obey equation (16). Hence any other choice of $F(t)$ is not consistent with this information on iodine in the thyroid.

In the fitting of a continuous function to the experimental curve of $M(t)$, many choices of functions are possible which will fit the data within the experimental error. For example, the data of Gellhorn, Merrell and Rankin (1944) could have been approximated by a polynomial in t. There is, of course, no procedure for insuring that different experimenters will fit the same type curves. In general, a good procedure is to try an exponential or combination of exponentials, for such equations are usually easy to integrate and may often be interpreted in terms of combinations of first order reactions. In addition, simplicity of representation is also to be preferred.

The integral equation procedure as presented in the paper reveals that we can set up a description of metabolizing systems which will define explicitly for such systems a rate function and a metabolizing function. In discussing such systems, experimenters would find themselves on common ground if some such formulation were accepted. Then a statement that the data are approximated by some continuous function as $M(t)$, described by a rate function, $R(t)$, and metabolizing function $F(t)$, would have explicit meaning in terms of equation (1). The tracer technique is well suited for the determination of $M(t)$. Conversely the integral equation formulation seems advantageous for correlating and integrating tracer studies in metabolizing systems. The procedure seems espe-

cially suggestive in studies involving doubly-tagged tracer molecules.

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STUDIES OF PURINE METABOLISM[1]

GEORGE BOSWORTH BROWN

Purine chemistry dates back to 1776 when Scheele discovered uric acid in human urine. This was followed a century later by the discovery of guanine (Fig. 1) by Unger (1846), of nucleic acids by Meischer (1871) and of adenine by Kossel (1885). The recognition of an association of purines and nucleic acids with the nuclei of cells led to many studies of the distribution and the functions of the purines. Rose has pointed out, in his comprehensive review (1923) of the early studies on purines, that attempts to study the distributions and functions of the purines are among the earliest metabolic studies on record. The fact that the animal organism does not require the presence in the diet of preformed purines or pyrimidines for the formation of nucleic acids made difficult the application of

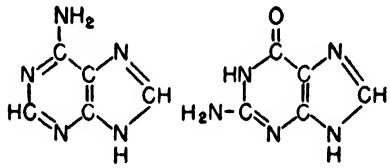

FIG. 1. Purines occurring in nucleic acids.

many of the classical biochemical methods to the study of their metabolism.

INITIAL TRACER STUDIES

The first application of the isotope tracer technique to the study of purine metabolism was that of Barnes and Schoenheimer (1943) who showed, both in pigeons and in rats, that dietary ammonia nitrogen is rapidly incorporated into the purines and pyrimidines of the nucleic acids of the internal organs. Plentl and Schoenheimer (1944), followed this with an investigation of the utilization of dietary purines and pyrimidines labeled with isotopic nitrogen. They fed isotopically labeled guanine, as well as the labeled pyrimidines, uracil and thymine (Fig. 2). They found that the guanine was not incorporated into the nucleic acids of pigeons or of rats. The ingested guanine was absorbed and was catabolized to uric acid or allantoin, the urinary end-products of purine metabolism in these species. Nor did the two pyrimidines tested lead to the formation of nucleic acids but they were metabolized to urea and to ammonia. That this purine and these two pyrimidines are not in a dynamic equilibrium with the guanine, uracil and thymine of nucleic acids was a

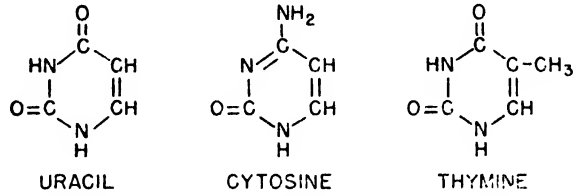

FIG. 2. Pyrimidines occurring in nucleic acids.

surprising finding. In fact these compounds, although they occur as components of the nucleic acids, behave as though they are end-products of nucleic acid metabolism in that they enter only into further catabolic reactions.

ADENINE AS A NUCLEIC ACID PRECURSOR

The other purine found in nucleic acids, adenine, is present not only in nucleic acids but also plays a role as a constituent of a number of enzymes and of the adenosinetriphosphate of muscle. In addition, adenine, its nucleoside, and nucleotides show marked physiological and pharmacological effects not shown by guanine and its derivatives. It seemed worthwhile to investigate the possible differences between the metabolic fate of adenine and that of guanine.

Adenine, labeled with an excess of isotopic nitrogen in the pyrimidine ring, was originally synthesized (Brown, Roll, Plentl and Cavalieri, 1948) by the method of Baddiley, Lythgoe and Todd (1943), although we are now using somewhat modified conditions (Fig. 3) (Cavalieri, Tinker and Brown, un-

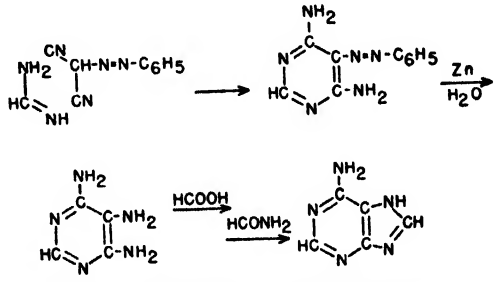

FIG. 3. Synthesis of labeled adenine.

pub.). One nitrogen containing 32 atom percent excess N^{15} was introduced in the formamidine. The synthesis from this compound resulted in the intro-

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duction of 16 atom percent excess N^{15} in each of the 1 and 3 nitrogens of the purine ring of adenine, about 6.4 atom percent excess average N^{15} content for the nitrogen of the molecule.

This adenine was fed to adult male Sherman strain rats. To insure against negative results due to insufficient adenine it was planned to first administer the compound in as large an amount as was compatible with the known toxicity (Allen and Cerecedo, 1933; Raska, 1946). Preliminary experiments indicated that rats of this strain could be fed up to 250 mg. of adenine per kilo of body weight per day for a week without the appearance of any symptoms of toxicity other than a moderate diuresis.

TABLE 1. FEEDING OF ISOTOPIC ADENINE

Labeled adenine fed to rats as the hydrochloride, equivalent to: A, 200 mg.; B, 27 mg. of adenine per kg. per day for 3 days

	A		B	
	Atom % excess N^{15}	Calc. on basis of 100% N^{15} adenine fed	Atom % excess N^{15}	Calc. on basis of 100% N^{15} adenine fed
Adenine (dietary)	6.29	100.	6.29	100.
Sodium nucleic acids	0.386	6.1		
Copper purines			0.23	
Purine hydrochlorides			0.23	
Adenine	0.857	13.7	0.34	5.4
Guanine	0.513	8.2	0.20	3.2
Silver pyrimidines	0.000	0.0		
Adenosinetriphosphate	0.161	2.6	0.002	0.03
Allantoin	1.70	27.0	0.348	5.53
Ammonia	0.02	0.32		
Urea	0.018	0.29	0.003	0.05
Muscle protein			0.00	0.0

In the first feeding experiment with labeled adenine, the adenine was fed at a level of 200 mg. per kilo of body weight per day for a period of three days. The sodium nucleic acids were isolated from the total viscera by sodium chloride extraction. The free nucleic acids were then prepared and from these the purines were isolated. The mixed pyrimidines were also isolated from the nucleic acids via their silver salts. Adenosinetriphosphate was isolated from the leg and back muscles and the thrice reprecipitated dibarium ATP contained an average of 2.7 moles of phosphorus per mole of adenine. Allantoin, urea and ammonia were also isolated from the urines.

The isotope content of these isolated products (Table 1) showed that the isotopic nitrogen of the dietary adenine was incorporated into the nucleic acids. It appeared not only in the adenine isolated from the nucleic acids but also in the guanine. In the first experiment, a total of 13.7 percent of the adenine of the nucleic acids was derived from the dietary adenine in the three-day period and a total of 8.2 percent of the guanine nitrogen was derived from the isotopic nitrogen of the dietary adenine during this period. That the pyrimidines contained no isotopic nitrogen showed that dietary adenine is not a precursor of pyrimidine nitrogen. The adenosinetriphosphate contained a definite but much lower percentage of isotopic nitrogen. Thus, this nucleotide may also be derived from dietary adenine, but at a slower rate.

In the case of the urinary allantoin, 27 percent of its nitrogen was derived from the dietary adenine. This indicates that nearly twice as much of the allantoin as of the nucleic acid-adenine was formed from dietary adenine, and is evidence for the existence of a direct oxidation of adenine to allantoin, a pathway which does not involve its prior incorporation into nucleic acids. The small amount of isotopic nitrogen in the ammonia and urea demonstrates that the purines are not degraded to either ammonia or to urea.

The first level at which adenine was fed, 200 mg. per kilogram per day, is abnormally high and approaches the level at which toxicities might occur. Therefore, a second experiment was carried out in which the amount of adenine fed per day was well below the amount of purines normally metabolized by the animals. From the data of Ackroyd and Hopkins (1916) it may be calculated that rats on a bread and milk diet excreted 262 mg. of allantoin per kilogram per day, while Leone (1945) found 60 to 140 mg. of allantoin produced per kilogram per day on diets stated to be purine free. For this experiment, a level of 27 mg. per kilogram per day was chosen as being well below the amount of purine normally turned over per day. In this experiment the copper purines isolated from the tissues and the mixed purine hydrochlorides isolated from the crude nucleic acids contained the same atom percent excess N^{15}. The isotope content of the adenine and guanine isolated showed that at this lower level there was a more efficient utilization of the dietary adenine for the formation of nucleic acids. Thus, although the amount of adenine in the diet was only 13.5 percent of that in the first experiment, the absolute amount of isotopic nitrogen in the purines of the nucleic acid was 39 percent of that in the first experiment. It is significant that in each experiment the nucleic acid-adenine and the nucleic acid-guanine were derived from the dietary adenine in the same ratio: that is, 1:0.60 and 1:0.59, and we have yet a third experiment in which this ratio proved to be 1:0.57.

At the lower level of intake of adenine the value for the isotope content of the ATP is practically normal. This behavior of the adenine moiety of ATP parallels the extremely low uptake of ammonia nitrogen in ATP (Barnes and Schoenheimer, 1943; Kalckar and Rittenberg, 1947). The turnover of the adenylic acid portion of the ATP is very slow under

normal conditions. The markedly greater incorporation of adenine into ATP when the animals were "flooded" with dietary adenine may be related to the toxicity of large doses of adenine, and this isolated observation should be confirmed.

Because of the striking difference between the results with adenine and the original observations with guanine we considered it advisable to repeat the feeding of guanine under our experimental conditions. The guanine was synthesized (Fig. 4) from isotopically labeled guanidine. It must be remembered that here, the 2-amino group, as well as the 1 and 3 nitrogens of the pyrimidine ring, contains an excess of isotopic nitrogen.

This guanine was fed at a level equivalent, on a molar basis, to the higher level at which adenine had been fed. The results (Table 2) completely confirmed those of the original investigators, that is, there was no utilization of the guanine for the formation of nucleic acids and it was extensively oxidized to allantoin. The labeled 2-amino group, which is lost in the oxidation of guanine to allantoin, contributed isotopic ammonia to the body pool which in turn gave the urea an appreciable isotope concentration. The ratio between the isotope level in the urea nitrogen and the trace found in the nucleic acid nitrogen was of the order found after the feeding experiments with isotopic ammonia.

TABLE 2. FEEDING OF ISOTOPIC GUANINE

Guanine fed as the sulfate, equivalent to 224 mg. of guanine per kg. per day for 3 days. This is equivalent on a molar basis to the higher level at which adenine was fed

	Atom % excess N^{15}	Calc. on basis of 100% N^{15} in guanine fed
Guanine (dietary)	6.40	100.
Sodium nucleic acids	0.009	0.14
Copper purines	0.00	0.0
Allantoin	2.02	31.9
Urea	0.115	1.80

END PRODUCTS OF PURINE METABOLISM

In man and apes the end-product of purine metabolism is uric acid. Birds and reptiles not only excrete uric acid as an end-product of purine metabolism but also utilize it for disposal of ammonia nitrogen. In other mammals, including the rat, allantoin, which is an oxidation product of uric acid, serves as the end-product of purines. The allantoin which was isolated from the urines after the feeding of adenine was degraded by reductive splitting with hydriodic acid, and the hydantoin obtained was analyzed. The hydantoin nitrogens were found to contain the same percentage of isotopic nitrogen as the whole allantoin molecule, which indicates that the isotopic nitrogen, originally in only the 1 and 3 nitrogens of the adenine, had now become uniformly distributed between the urea and the imidazole moieties of the allantoin molecule. There is evidence in the literature, dating back to the time of Emil Fischer, to suggest that uric acid, when oxidized *in vitro*, reaches allantoin via a symmetrical intermediate (Fig. 5). Either ring of this intermediate hydroxyacetylene diureide carboxylic acid may be cleaved to produce allantoin with either given

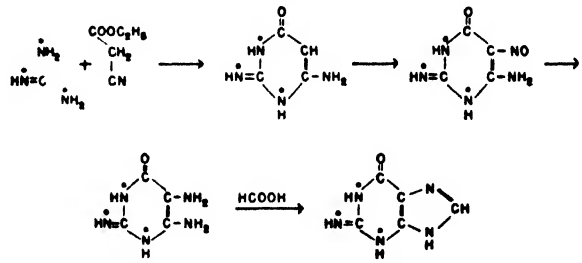

FIG. 4. Synthesis of labeled guanine.

pair of nitrogen atoms in either the urea or the hydantoin moieties. Statistically the product isolated will have its isotopic nitrogen uniformly distributed.

Confirmation of this hypothesis came from the degradation of unsymmetrically labeled uric acid. The uric acid was synthesized (Cavalieri, Blair and Brown, 1948) containing isotopic nitrogen in positions 1 and 3. Samples of the allantoin obtained from this synthetic uric acid by alkaline permanganate oxidation were degraded (Cavalieri and Brown, 1948) to hydantoin and to potassium oxonate. The analyses of these products indicated that each contained exactly the same concentration of isotopic nitrogen and thus that the isotopic nitrogen had become uniformly distributed throughout the nitrogen of the allantoin. Another sample of uric acid was oxidized to alloxan. That this product had resulted by direct removal of the imidazole moiety while the pyrimidine ring remained intact was demonstrated by analysis which showed that this alloxan contained double the concentration of isotopic nitrogen of the original uric acid.

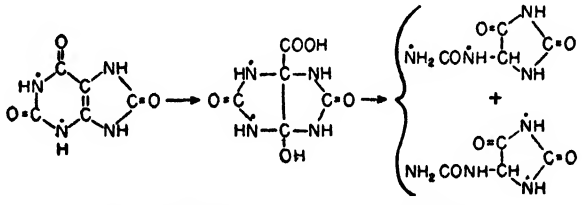

FIG. 5. Oxidation of uric acid to allantoin.

A sample of uric acid, labeled in the 1 and 3 positions, has also been fed to rats (Brown, Roll and Cavalieri, 1947). The fact that the allantoin which was produced in the *in vivo* oxidation (Table 3) also yielded hydantoin of the same isotope content

showed that the isotope was uniformly distributed in this sample of allantoin. Thus the oxidation of uric acid in the intact animal must also proceed via a symmetrical intermediate and it is probable that the redistribution of the nitrogens of the adenine and guanine took place at this stage in their oxidation.

This experiment also demonstrated that no uricolysis to urea or ammonia took place and that there was no utilization of uric acid for the formation of other purines.

Precursors of Purines

With the finding that adenine is transformed into guanine, the question of the origin of purines becomes a question of the precursors of adenine. Recently a great amount of information as to the smaller molecules which are precursors of purines has come from the study of the precursors of uric acid in pigeons. Buchanan, Sonne and Delluva (1948), using various precursors labeled with heavy carbon, have shown that the carboxyl carbon of acetic acid and formic acid participate in the synthesis of the ureide carbons, carbons 2 and 8. They have shown that the 6-carbon is derived from respiratory CO_2 and that the 4-carbon can be derived from the α-carbon of lactate while the 5-carbon can be derived from the β-carbon of lactate. They have also shown that the carboxyl-carbon of glycine is a more immediate precursor of the 4-carbon of uric acid, while at about the same time Shemin and Rittenberg (1947) showed, in man, that the 7-nitrogen of uric acid was specifically derived from the nitrogen of glycine. These two results indicate that the 4-, 5- and 7-atoms of the uric acid molecule come quite directly from glycine.

Subsequently Abrams, Hammarsten and Shemin (1948) have cultivated yeast in the presence of N^{15} labeled glycine and they have found a specific uptake of the labeled glycine in both the adenine and the guanine isolated from it. By degrading the isolated guanine to glycine which represents the 4-, 5- and 7-atoms, they showed that the N^{15} labeled glycine was a specific precursor of the 7-nitrogen of the guanine. In this experiment, and in the additional experiments reported by Dr. Hammarsten today, it was found that the uptake of the isotopic nitrogen from the isotopically labeled glycine was up to twice as much in the guanine as it was in the adenine.

Table 3. Feeding of Isotopic Purines

	Hypoxanthine[a]		Xanthine[b]		Uric Acid[c]		Isoguanine[d]	
	Atom % excess N^{15}	Calc. on basis of 100% N^{15} in hypoxanthine fed	Atom % excess N^{15}	Calc. on basis of 100% N^{15} in xanthine fed	Atom % excess N^{15}	Calc. on basis of 100% N^{15} in uric acid fed	Atom % excess N^{15}	Calc. on basis of 100% N^{15} in isoguanine fed
Dietary Purine	4.34	100.	5.37	100.	16.0	100.	5.40	100.
Nucleic acids	0.006	0.12	0.006	0.11			0.012	0.20
Copper purines			0.003	0.06	0.000	0.00		
Purine hydrochlorides	0.002	0.05					0.007	0.13
Purines from muscle	0.004	0.09						
Allantoin	0.333	7.7	1.32	24.6	5.37	33.4	0.890	16.5
Hydantoin (from allantoin)					5.34			
Urea	0.009	0.21	0.015	0.28	0.006	0.04	0.057	1.1
Ammonia	0.011	0.25	0.029	0.54			0.025	0.47

[a] Hypoxanthine, 26 mg. per kg. per day for 3 days; equivalent to the lower level at which adenine was fed.
[b] Xanthine, 105 mg. per kg. per day for 4 days; equivalent to 3.5 times the lower level at which adenine was fed.
[c] Uric acid, 249 mg. per kg. per day for 3 days; equivalent to the higher level at which adenine was fed.
[d] Isoguanine, 230 mg. per kg. per day for 3 days; equivalent to the higher level at which adenine was fed.

We have consistently found, with labeled dietary adenine, that the nucleic acid-guanine contains about 40 percent less isotopic nitrogen than the nucleic acid adenine. The greater uptake in the guanine of N^{15} from administered glycine seems to be too much to be accounted for by an additional specific uptake of glycine nitrogen into the 2-amino group, and requires further exploration.

Among amino acids other than glycine which might be precursors of purines the long popular theory that arginine and histidine might be specific precursors was largely based upon the formal analogy due to the presence of the guanidino and imidazole moieties. These amino acids have been excluded by the negative results of Bloch and Schoenheimer (1941), who fed rats isotopically labeled arginine, and of Tesar and Rittenberg (1947), who fed histidine labeled in the imidazole ring.

Among the larger molecules which might be di-

rect precursors of adenine there is considerable evidence in the literature to support the postulate that the immediate precursor is hypoxanthine. Örström, Örström and Krebs (1939) have shown that hypoxanthine may be formed in pigeon liver slices under certain conditions, and they suggested that hypoxanthine is an intermediate in uric acid formation by the intact organism. Sonne, Buchanan and Delluva (1948) have stated that their data are in accord with this postulation. In the case where hypoxanthine and adenine bear a 5-phosphoribose in the 9-position, that is, in the case of muscle inosinic acid and muscle adenylic acid, it has been shown (Kalckar and Rittenberg, 1947) that the 6-amino group of adenylic acid takes up isotopic nitrogen even more rapidly than glutamic acid, although somewhat less rapidly than the amide-N of protein. Thus these ribosides of hypoxanthine and adenine are rapidly interconvertible. Also, of the 45 adenine-requiring mutants of *Neurospora crassa* tested by Mitchell (1946), 43 will utilize hypoxanthine in lieu of adenine for their purine requirement.

Other support for the consideration of hypoxanthine as a precursor comes from the character of a compound which accumulates in the cultures of certain bacteria when purine synthesis is competitively inhibited (Shive and Roberts, 1946) by sulfonamides. This compound has been isolated by Stetten and Fox (1945) and Shive and co-workers (1947) have identified it as 5(4)-amino-4(5)-imidazolecarboxamide. It has been shown (Ravel, Eakin and Shive, 1948) that the addition of glycine or threonine to the medium stimulates the production of this imidazole and these authors have suggested that it is the immediate precursor of hypoxanthine. Both the work of Sonne, Buchanan and Delluva, showing that formic and acetic acids are precursors of the 2-carbon of uric acid and that of Shive (1948) showing that formyl folic acid is a most potent antagonist of this sulfonamide inhibition, favor the hypothesis that it is this imidazolecarboxamide plus formic or acetic acid which leads to hypoxanthine, which would in turn be the immediate precursor of adenine (Fig. 6).

With this much evidence favoring the possibility of hypoxanthine serving as a precursor of adenine, we are in the process of testing isotopically labeled

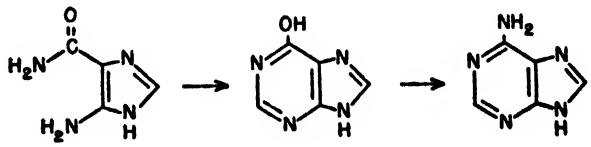

FIG. 6. Relationships of 5(4)-amino-4(5)-imidazolecarboxamide, hypoxanthine and adenine.

hypoxanthine (Getler, Roll and Brown). Hypoxanthine was prepared by the deamination of isotopic adenine. Hypoxanthine prepared thus contains small amounts of adenine and, to make certain that a false result was not obtained because of traces of adenine present, the dietary sample of hypoxanthine used was freed of adenine by a preparative counter-current distribution, and was characterized by an analytical counter-current distribution and shown to contain less than one percent of adenine.

It should be mentioned here that routine use has been made of the Craig counter-current distribution technique for the qualitative characterization and the determination of homogeneity of purines and pyrimidines encountered in the course of biological and chemical work (Tinker and Brown, 1948). For compounds such as these, which are at best difficult to characterize, the ability to determine homogeneity with adequate precision has made the counter-cur-

TABLE 4. DISTRIBUTION CONSTANTS FOUND FOR SOME PURINES AND PYRIMIDINES
System: *n*-butanol—1 M phosphate buffer of pH 6.5

		Partition coefficient	Concentration in Tube O	Absorption maxima
			mg. per cc.	mμ
Purines	Adenine	2.77	2.65	260
		2.14	0.12	
	Guanine	0.45	0.11	247, 274
	Hypoxanthine	0.54	2.00	250
	Xanthine	0.46	0.15	269
	Isoguanine	0.28	0.56	239, 286
	Uric acid	0.11	Saturated	240, 293
	2-thioadenine	0.48	0.8	228, 256–62, shoulder at 283
	2,6-diaminopurine	1.21	0.46	250, 281
Pyrimidines	Thymine	1.11	2.00	265
	Uracil	0.401	2.00	260
		0.400	0.56	
	Cytosine	0.207	2.00	267
		0.206	0.50	

rent procedure a very valuable tool. The range of distribution constants found is indicated in Table 4. The solvent pair used is one which is optimal for the characterization of adenine-guanine mixtures, and is equally satisfactory for adenine-hypoxanthine mixtures.

The adenine-free hypoxanthine has been fed to rats only at a level of 26 mg. per kilogram of body weight per day which is equivalent to the lower level at which adenine was fed. The results obtained

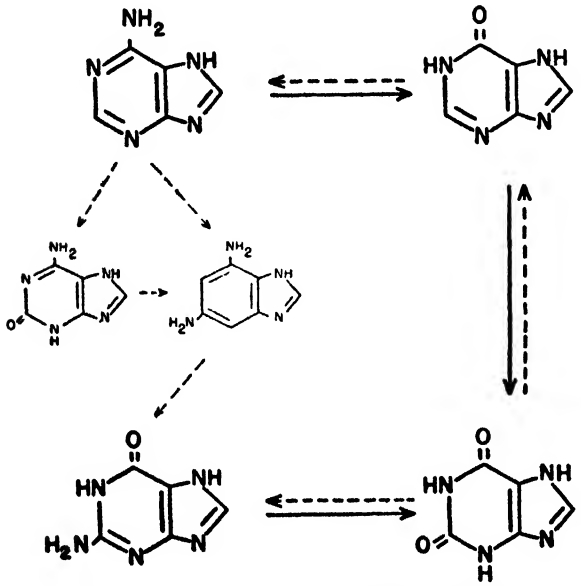

FIG. 7. Hypothetical relationships of adenine, isoguanine and guanine.

(Table 3) showed that at this level the compound is not a precursor of nucleic acid purines nor of muscle adenylic acid. This hypoxanthine was catabolized to allantoin to a somewhat greater extent than was an equivalent amount of adenine. Thus, although some derivative of hypoxanthine may be a precursor of nucleic acid purines, the free purine does not so serve.

We have also prepared isotopically labeled xanthine by the deamination of guanine and this purine was fed to rats at a somewhat higher level. Here too, the only fate of xanthine was oxidation to allantoin (Table 3). In agreement with the results obtained with adenine, hypoxanthine and uric acid, there was no appreciable amount of isotopic nitrogen in the urea and ammonia and these data confirm our opinion that the considerable amount of N^{15} which was found in the urea after the feeding of guanine was derived only from the labeled 2-amino group and did not come from the nitrogen of the purine skeleton.

CONVERSION OF ADENINE TO GUANINE

To gain some insight into the mechanism by which the conversion of adenine to guanine is accomplished, a determination of the position of isotopic nitrogen in a sample of guanine which had been formed *in vivo* from adenine was carried out. The guanine was deaminated to xanthine with the elimination of the 2-amino group. Another sample was oxidized to guanidine which represents the 1 and 3 nitrogens of the pyrimidine ring as well as the 2-amino group. The results of the isotope analyses showed that each of these products contained all the isotopic nitrogen of its guanine precursor. Therefore the isotopic nitrogen was still in the 1 and/or 3 positions of the purine ring of the guanine, and the adenine was probably converted into guanine with the retention of the intact purine skeleton.

In a consideration of possible mechanisms for the conversion of adenine to nucleic acid-guanine certain possibilities are open. If free purines and not derivatives, such as their ribosides, are involved in this transformation, xanthine and hypoxanthine are not among the possible intermediates. Thus deamination of adenine as the first step in the transformation is eliminated and the alternative of the primary step being an oxidation of adenine in the 2-position becomes a possibility to be considered. This oxidation would lead to 2-hydroxyadenine, or isoguanine, and although isoguanine is not a common purine it does occur in nature (Cherbuliez, 1932; Spies, 1939) as the base of the nucleoside, crotonoside, of the croton bean. In addition, evidence that a direct oxidation of adenine may occur *in vivo* is derived from the fact that after the feeding of adenine, the deposition in the kidneys of the 6-amino-2,8-dioxypurine has been shown to occur (Minkowski, 1898; Nicolaier, 1902; Ebstein and Bendix, 1904). An oxidation of adenine to isoguanine, followed by amination to 2,6-diamino purine, with subsequent deamination at the 6-position to yield guanine is one possibility for a conversion of adenine to guanine (Fig. 7).

We have devised syntheses suitable for the incorporation of isotopic nitrogen into isoguanine and 2,6-diamino purine, and have so far prepared and tested isotopically labeled isoguanine. The synthesis of isoguanine (Bendich, Tinker and Brown, 1948) involved some interesting chemistry. The most obvious approach seemed to be to follow the general procedure of Traube (1900), and prepare 2-thioadenine and to then replace the sulfur by oxygen through treatment with chloroacetic acid. The synthesis of the initial 4,6-diamino-2-thiopyrimidine from thiourea and malononitrile afforded a good introduction of the isotopic nitrogen, since thiourea may be readily prepared from isotopic ammonia and cyanogen bromide.

Desulfurization of a thio compound of this type by treatment with hot chloroacetic acid is a quite general method, but, in this instance the 2-thioadenine yielded a remarkably stable carboxymethylthiol derivative and the desulfurization was not accomplished. After investigating several possibilities (Fig. 8) the synthesis was finally completed by the removal of sulfur from the initial 4,6-diamino-2-thio-

pyrimidine, with subsequent nitrosation and reduction to introduce the 5-amino group, followed by formation of the imidazole ring.

The feeding of the labeled isoguanine did not lead to any uptake of isotopic nitrogen in the purines of the nucleic acids. An interesting finding was that, even though this is not a "normal" purine, it was quite effectively oxidized to allantoin. In addition, the isotopic nitrogen found in the urinary urea was considerably more than the trace found in the urinary ammonia. It would have been possible for some urea to have arisen directly from the labeled allantoin present in the urine by bacterial oxidation to allantoic acid, followed by hydrolysis to urea. However, the isotope values obtained from three separate isolations, one made after the urine had

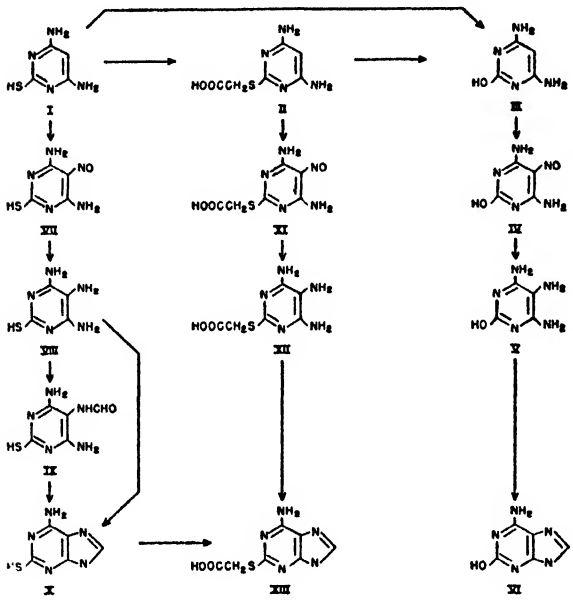

FIG. 8. Synthesis of 2-thioladenine and 2-hydroxyadenine (isoguanine).

stood for about three months without showing any signs of bacterial contamination, were in agreement and were more than twice the value for the ammonia. These data indicate that isoguanine was, to a considerable extent, oxidized directly to yield urea from either or both of the labeled nitrogens. This is the only purine which has been found to be catabolized to urea to any significant extent.

USE OF ADENINE TO LABEL NUCLEIC ACIDS

In our first experiments with adenine and during these experiments where we have been exploring the fate of other purines, no attempt was made to separate the two types of nucleic acids. We have now carried out an experiment in which labeled adenine was fed and the relative uptake in the pentose nucleic acids (PNA) and the desoxypentose nucleic acids (DNA) was determined.

TABLE 5. ANALYSES OF NUCLEIC ACID FRACTIONS

Total nucleic acids from viscera of five rats and fractions obtained by Schmidt-Thannhauser procedure

	Pentosenucleic acids, mgs.	Desoxypentose-nucleic acids, mgs.
Desiccated and defatted tissue	749	560
DNA fraction	0 to 20	400
PNA fraction	781	0 to 40

Labeled adenine was fed to five adult male rats at a level of 27 mg. per kilo of body weight per day for ten days (Brown, Petermann and Furst, 1948). The separation of the two types of nucleic acids from the total viscera was carried out by a large scale application of the Schmidt-Thannhauser (1945) method, which depends upon the marked ease of depolymerization in alkali of the pentose nucleic acids (PNA) in contrast to the relative stability of the desoxypentose nucleotide polymer under the same conditions. Each fraction was analyzed (Schneider, 1946) for phosphorus content, for its PNA content by the orcinol method and for its DNA content by the diphenylamine method. Each nucleic acid fraction was shown to be free of the other within the experimental limits of the methods (Table 5). The purines isolated from the nucleic acid fractions were characterized by counter-current distribution and each was shown to be free of the other.

Analyses (Table 6) of the adenine and guanine showed that in the PNA fraction 15.9 percent of the adenine and 9.1 percent of the guanine originated from the dietary adenine. In the purines of the DNA fraction the low isotope concentrations indicated a replacement which is only 3.5 percent of that obtained in the PNA fraction. Although the actual determination of the amount of PNA present in the DNA fraction had given a value of zero, the uncertainty involved in the determination of traces of PNA in the presence of large amounts of DNA is such that we must allow for an error of possibly

TABLE 6. ISOTOPE CONTENT OF PURINES ISOLATED FROM FRACTIONATED NUCLEIC ACIDS

	Pentose nucleic acids		Desoxypentose nucleic acids	
	Atom % excess N^{15}	Calc. on basis of 100% N^{15} in adenine fed	Atom % excess N^{15}	Calc. on basis of 100% N^{15} in adenine fed
Adenine (dietary)	6.29	100.	6.29	100.
Adenine picrate	0.634		0.029	
Adenine	1.00	15.9	0.035	0.55
Guanine	0.572	9.1	0.024	0.32

5 percent. At that time we were using the orcinol procedure for the determination of PNA and this reagent gives about 10 percent as much color with DNA as it does with PNA. A correction of the PNA value, based upon the value found for the DNA, must be applied. Consequently it is impossible to say that the N^{15} uptake observed in the DNA fraction may not be partially or entirely ascribed to contaminating PNA. In fact if the N^{15} uptake in the DNA fraction should represent only PNA contamination it is a more certain measure of the PNA content of that fraction than is the orcinol test.

It is even conceivable that there is a fundamental difference in the origin of the purines of PNA and of DNA. However, if we accept the N^{15} uptake in the DNA purines as a valid figure and calculate the ratio of uptake of adenine in the PNA-purines to the DNA-purines, we find the ratio of turnovers to be 29:1, which is a minimum value and may be considerably greater.

It is difficult to compare this observed ratio of turnover of PNA-purines:DNA-purines with the available data on the phosphorus turnover of the two types of nucleic acids because of the varying biological conditions and chemical procedures used by different investigators. Brues, Tracy and Cohn (1944) reported, using P^{32}, that in normal rat liver the ratio of the turnovers of PNA-phophorus: DNA-phosphorus were 5.2:1 in three days, and 5.9:1 in 8 days, although Brues (1947) has commented that higher ratios have subsequently been obtained. Hammarsten and Hevesy (1946) found that for total rat viscera the turnover ratio PNA-P: DNA-P was 1.66:1 after two hours, and for individual organs was: liver 33:1, spleen 3:1, and intestine 2:1. Davidson and Raymond (1948) found the ratio for PNA-P:DNA-P to be 7:1 in liver after 2 to 4 hours; and in three day experiments with administered isotopic ammonia they found higher PNA-N:DNA-N turnover ratios, although the low isotope levels encountered made the calculation of the exact ratio unsatisfactory.

The PNA-purine:DNA-purine ratio of 29:1 (or greater, depending upon the extent to which the N^{15} found in the DNA fraction is due to true DNA turnover and to which it is due to PNA contamination) found for total viscera after feeding labeled adenine for 10 days is much higher than any comparable figures for phosphorus turnover and emphasizes the extremely slow rate of turnover of the nucleic acid-purines in the resting nucleus. If, as the available data indicate, the ratios of turnover of PNA-purines: DNA-purines and PNA-P:DNA-P are different, at least some portion of the phosphate moieties of the nucleic acid may be exchanged without the purines of C-N skeleton being affected.

It might be mentioned in conclusion that we have also prepared and tested the pyrimidine cytosine (Bendich and Brown) labeled in the ring nitrogens. This is the only one of the five purines and pyrimidines occurring in nucleic aids which had not yet been tested as to its role as a precursor of nucleic acids, and it too proves not to be a precursor of nucleic acid nitrogen.

Except for the unique case of adenine, the free purines (and pyrimidines), once they are formed, enter only into further degradation reactions and are thus in the class of catabolic products of nucleic acids. Free guanine, although its derivatives serve as structural components of nucleic acids, must be included here along with hypoxanthine.

The *in vivo* syntheses of pyrimidines must take place in the form of some derivative, possibly the ribosides. The conversion of the adenine moiety to the guanine moiety also probably takes place in some conjugated form. Possibly the *in vivo* synthesis leading to adenine also proceeds via a derivative, but in this case some intermediate must be capable of being formed from free adenine.

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DISCUSSION

D. W. WILSON: We have studied the incorporation of C^{14} in nucleic acid of the rat after injection of isotopic sodium bicarbonate. This has led to the incorporation of C^{14} in adenine with small amounts appearing in #6 carbon of guanine and the ureide carbon of uracil.

STUDIES OF THE OVER-ALL CO_2 METABOLISM OF TISSUES AND TOTAL ORGANISMS

AUSTIN M. BRUES AND DONALD L. BUCHANAN

Prior to the time when carbon^{14} became readily available, there were no isotopes of long half-life in common use and of comparable usefulness in biological science. Because of the potential harmfulness of long-lived isotopes deposited in the human body, there has, therefore, been some concern over the possibility of chronic human carbon^{14} poisoning. This concern has not been alleviated by the findings of Bloom, Curtis and McLean (1947) who showed by autoradiographic means that a portion of the labeled bicarbonate injected into rats is still present in the bones after a period of several months.

An attempt to assess this hazard may be made on theoretical grounds if one makes the simplifying assumption that all carbon^{14} introduced into the body becomes permanently stored. If the highest amount of radiation to which the body may safely be exposed is taken as 0.1 roentgen per day, it can be shown that about 2.26 millicuries of C^{14} distributed evenly through the tissues of a 70 kilogram man would yield the daily equivalent of ionizing radiation (Morgan, 1947). In view of the short range of the beta particles in tissue and the possibility of selective concentration in certain loci, an additional factor should be used. If all of the retained carbon were in the skeleton, for example, the allowable amount would be about one-third millicurie.

It may be that additional factors should be included because of our ignorance of the exact nature of the process whereby ionizing radiations induce malignant tumors. It has been shown (Lisco, Finkel and Brues, 1947) that strontium^{89}, if injected at intervals in such a way as to compensate for the decay of the radioelement, is about one-tenth as active in inducing osteogenic tumors (on a millicurie basis) as radium. This finding, not predictable on a physical basis, suggests that a safer figure might be reached by using this observation and the established radium tolerance (0.1 microcurie). The permissible amount of strontium^{89} deposited in the human skeleton would then be ten times that established for radium, or one microcurie, and that of carbon^{14}, which has approximately one-tenth the beta-ray energy of strontium^{89}, would accordingly be ten microcuries. Since C^{14} will not be confined to bone, 30 microcuries has been suggested tentatively as a maximum permissible retained dose (Brues, 1948). In the hypothetical case of a metabolically inert compound in high local concentration, a further reduction of this figure might be necessary.

The discovery of carbon dioxide fixation by Wood and Werkman (1935) without the aid of isotopes prefaced a series of investigations on the mechanisms and pathways involved in this process. These experiments were performed in several laboratories using stable C^{13} and short-lived C^{11}, and more recently, C^{14}. The information thus gained has given us a very substantial outline of the early pathways that carbon dioxide may take after its reduction and has served to indicate a variety of ways in which carbon may be utilized in the synthesis of various compounds. Thus, numerous workers have found that injected or fed labeled bicarbonate appears in liver glycogen and bone as well as in circulating bicarbonate and expired air, and in many other compounds. But aside from establishing the fact of fixation of carbon, the present information is of little help in assessing the degree and duration of tissue exposure following, for example, the inhalation of $C^{14}O_2$.

It would be a mistake to suggest that the chief hazard in the use of C^{14} is necessarily the fixation and storage of carbon dioxide. It is almost a certainty that exposure to an equal amount of the isotope in an organic molecule capable of being metabolized would result in greater retention. But the highest specific activities used experimentally are usually in the form of carbonate. The intangibility of the gas, the tendency of inorganic carbonates to exchange with atmospheric carbon dioxide, and the difficulty of satisfactory monitoring make it necessary to rely on good technique and good laboratory design. Experiments which demonstrate the dynamic principles involved in the uptake, retention and excretion of carbon dioxide must be performed before we can make a rational estimate of tolerance levels, and before we can specify equipment capable of detecting these levels.

A few general remarks can be made about the probable dynamics leading to retention of C^{14} administered as CO_2. In an ideal steady state, those compounds which are most rapidly regenerated will lose their carbon into the carbonate pool most rapidly, while the more stable compounds or groups will exhibit the most sluggish uptake. The studies of Armstrong, Schubert and Lindenbaum (1948) and those of Gould, Rosenberg, Sinex and Hastings (1948) indicate that, following brief exposure by intraperitoneal bicarbonate injection, the retention reaches a very low level after a few hours. This is in contrast to the rapid acquisition and slow surrender of calcium and related elements by bone which will be discussed by Dr. William Norris later in this symposium.

This ideal steady state is, of course, not always

FIG. 1. Chamber for exposure of mouse in a closed system.

FIG. 2. A Geiger-Mueller tube and counting rate meter in place.

attained in the living organism. Where tissue growth is occurring, there exists the possibility that carbon may be synthesized into compounds formed *de novo* which may, after synthesis, have a sufficiently slow rate of turnover effectively to lock the tracer into the compound. We have shown (Brues, Tracy and Cohn, 1944) that this occurs in the case of desoxyribose nucleic acid formed in regenerating liver, using P^{32}, and Davidson (1947) has verified this with isotopic nitrogen. Additional evidence pointing in this direction has been given in this symposium by Hammarsten and by Brown. Most tissue growth in the adult (*e.g.*, that in the blood-forming organs and surface membranes) is a replenishment of lost or excreted cells, so that a cellular steady state is maintained, and this type of growth may occur in tissue cultures (Brues, Rathbun and Cohn, 1944). The result, in terms of C^{14} retention, would be similar to that where compounds have a turnover time corresponding to that of the life of the tissue cell in question. Net growth would appear to be a condition more favorable for long-term carbon retention, such as occurs in the bones of the growing rat, or in embryos and young animals.

The experimental work to be detailed here is preliminary and gives only a semi-quantitative picture of the overall carbon dioxide metabolism after exposure to the gas or to bicarbonate. It represents the work of the authors and of Drs. Robert R. Newell, Asher J. Finkel and Lester F. Wolterink, and Miss Agnes Naranjo. We shall discuss the handling of carbon dioxide vapor in a closed system, its metabolism in an expanding or flushed system, and its retention by growing tissue in cultures.

The Closed System

In practice, exposure to radioactive carbon dioxide gas may occur under different conditions. A high-level exposure may be due to the sudden release of a large amount of gas in a ventilated environment. A chronic low-level exposure may be the result of the gradual evolution or leakage of the radioactive gas into a laboratory in such a way as to maintain a more or less constant concentration. Both of these conditions are difficult to duplicate experimentally and entail great wastage of the isotope because of the technical problems of radioactivity measurement at tissue tolerance levels and the constant dilution of the tracer in air by the quantity of carbon dioxide evolved by the animal.

A series of mice was studied in the closed system shown in Figs. 1 and 2. This consists of a sealed bell-shaped chamber containing the mouse, into which an end-window Geiger tube is inserted. In some experiments the tube was placed outside the chamber, which was covered with a thin, gas-tight nylon film (thickness 1 mgm./cm.^{2}). The tube was connected with a counting rate meter and the readings gave a continuous record of the concentration of $C^{14}O_2$ in the air surrounding the mouse. A drying agent was used in the chamber to prevent condensation of water vapor, which (in the case of the nylon film) could interfere seriously with the validity of the readings through absorption of C^{14} beta rays.

The chamber with the mouse was sealed and flushed with fresh air or five percent CO_2 until the beginning of the observation period, when the inlet and outlet cocks were closed and about 10 μc. $C^{14}O_2$ was introduced rapidly by the use of a mercury leveling device. Gaseous mixing was ordinarily complete within 30 seconds, following which the measured concentration fell exponentially to a level which was maintained within the limits of accuracy of measurements for at least one-half hour. The chamber was flushed at 30 minute intervals, to remove accumulated CO_2. With a mouse weighing 20 to 25 grams in a 770 ml. chamber, the concentration of carbon dioxide, starting from room air, did not exceed three percent. A mouse could survive in this closed system for more than 90 minutes.

Upon flushing the radioactive gas from the chamber at the end of a half-hour exposure period, $C^{14}O_2$ then returned from the mouse to the gas phase until a new level was reached. Following further flushings, such levels were reestablished.

In one typical experiment, 14 such washings were performed. During the initial period of exposure, the mouse removed about 30 percent of the 12 microcuries originally introduced. During the ensuing three flushings, about 80-90 percent of the estimated retained carbon dioxide was returned by the mouse to the chamber. After this time, a slow and relatively constant output of $C^{14}O_2$ was observed, ranging from 4 percent to 1 percent of the originally absorbed $C^{14}O_2$ per half-hour. Although none of the data have been adequate for purposes of plotting curves of loss of this "metabolic" fraction, it appears to exceed 10 percent of that originally taken up during a 30-minute exposure.

In Table 1, the data are given for a series of mice (female, not fasted) indicating the fraction of CO_2 taken up and the rate of uptake. In interpreting the data, it is necessary to consider the nature of the diffusion of CO_2 into the blood and body fluids in the light of our knowledge of the reverse process.

Obviously, although diffusion of CO_2 across the pulmonary barrier proceeds very efficiently, it would be impossible for all of the tidal CO_2 to gain access to the blood because of the "physiological dead space." Thus, just as the expired air has a lower CO_2 concentration than that of the alveolar air, the latter can never attain a concentration of $C^{14}O_2$ equal to that of the inspired air while any net inflow of $C^{14}O_2$ is occurring. It is likewise impossible for all of the alveolar $C^{14}O_2$ to enter the blood and be carried away through the circulation, since its passage into the blood must take place through an exchange of CO_2. The theoretical maximum uptake of $C^{14}O_2$ may be estimated by considering rates of air

and blood flow through the lung.

The alveolar CO_2 partial pressure is probably regulated quite closely throughout the respiratory cycle and is little influenced by the external air concentration up to four to five percent CO_2 in the air (Haldane and Priestly, 1922). The alveolar level may be taken as about 5.5 percent. A mouse breathing room air and excreting one cc. of CO_2 per minute has therefore passed about 18 cc. of air through the alveoli. If we take 58 and 53 volumes percent as the contents of the pulmonary arterial and venous bloods, respectively, the removal of five volumes percent from the blood indicates that 20 ml. of blood has traversed the pulmonary capillaries during this time. These, of course, represent average values from measurements taken over periods of time long enough to cancel out momentary fluctuations during the respiratory cycle. In the case cited, one volume of blood is exchanging with 0.9 volumes of alveolar air.

Because of the known rapidity of conversion of CO_2 to carbonic acid and bicarbonate in the presence of carbonic anhydrase and due to the binding of CO_2 by hemoglobin (Roughton, 1935), the incorporation of absorbed $C^{14}O_2$ into the blood carbonate system during passage of the blood through the pulmonary capillaries may proceed nearly to completion. Since the distribution ratio is about ten to one in favor of blood, complete mixing at the rates of air and blood flow given above would result in slightly over 90 percent of all $C^{14}O_2$ entering the alveoli becoming incorporated into the blood in the hypothetical case here cited.

In case the ventilation rate were increased out of proportion to the blood flow (as in an animal hyperventilating in a high external CO_2 concentration), one would expect the expired air to contain a higher proportion of the inspired $C^{14}O_2$ concentration, but the higher ventilation rate could still result in a more rapid rate of absorption of the isotope. The pulmonary blood flow may also be an important limiting factor (Jones, 1946).

The clearance rates as calculated from the data in Table 1 represent the amount of air cleared of $C^{14}O_2$, as measured by initial uptake, and will necessarily be less than the ventilation rate in accordance with the factors just mentioned. Inspection of the figures suggests that clearance proceeds very efficiently, although direct measurements are lacking. It is interesting to compare these figures with the values published by Guyton (1947), which show an average respiratory volume between 17 and 28 cc. per minute in mice of 20 gm. mean weight.

The uptake of carbon monoxide by man, which has been extensively studied (Forbes, Sargent and Roughton, 1945) shows a similar efficiency in its passage into the circulation. In two male subjects at rest, 60 and 67 percent of the carbon monoxide was cleared from the inspired air.

A steady state will be reached when the amounts of $C^{14}O_2$ leaving and entering the blood through the lung are equal. This will be determined by the specific activities of the blood CO_2 and the alveolar CO_2 (that of the external air being irrelevant except insofar as it affects the alveolar concentration). Since there is a net outflow of CO_2 from the blood, the steady state specific activity of blood will be less than that of the alveolar air by a ratio determined by the CO_2 outflow relative to the exchange rate. Since certain of these data have not been determined experimentally, the "volume of dilution" of $C^{14}O_2$ in the mouse during the 15 to 30 minute period is expressed in Table 1 as cc. of external air and will be seen to be about ten times the volume of the animal. Although it would be desirable to calculate from this the mass of carbon in rapid exchange with the alveolar air, one can see the impossibility of doing this without further knowledge of the processes involved. As in the experiments to be described below, it appears to be somewhat greater than the total CO_2-bicarbonate system (excluding bone carbonate).

TABLE 1. EXPERIMENTS IN CLOSED CHAMBER

Chamber volume in all cases 770 cc.

Number of Mice	Weight in grams		Half Time Early Uptake (Minutes)		Equilibrium % in mouse		Clearance rate cc. per minute	Volume of CO_2 dilution as cc. of air
	Mean	Range	Mean	Range	Mean	Range		
4	23.5	(22–26)	5.8	(3.5–6)	21.5	(20–24)	26.3	211
4	27.0	(27)	5.7	(3–6.5)	26.0	(18–34)	34.5	270
5	30.2	(29–32)	5.4	(4–6.5)	32.4	(28–38)	49.0	368

THE FLUSHED SYSTEM

Theoretical Treatment

Exposure to a large quantity of $C^{14}O_2$ gas is likely to occur with the knowledge of the exposed individual, who will take steps to reach a ventilated environment. This circumstance is approximated experimentally by a flushed or ventilated system. Analysis of the results is somewhat similar to the method described by Cohn and Brues (1945) for a tissue culture system containing P^{32}.

Consider a chamber, I, into which a known quan-

tity of radioactive gas, A, is introduced, and which is then ventilated at a constant rate with inert gas. (See Fig. 3.) If mixing is instantaneous, analysis of the gas in the chamber will show that at any time, t, the amount of the radioactive gas in the chamber, x, will be represented by the function,

$$x = Ae^{-t}, \qquad (1)$$

when the units of time are so chosen that the volume of gas withdrawn per unit time is equal to the volume of the chamber.

If a second chamber, II, be added to the system so that it in turn exchanges gas with chamber I (at any finite rate), chamber I will no longer be emptied according to (1), but will at first empty at a faster rate because the gas is leaving it by two pathways. However, after a time the concentration of radioactivity will become greater in II and chamber I will empty at a slower rate because it will be receiving radioactivity from II.

When a third complication is added to this system, namely that inert gas is made to enter chamber II, pass through it into chamber I, and out through the ventilating system, a model is set up which represents some of the elements of a biological situation. In this model which is illustrated in Fig. 3, the volumes of chambers I and II are represented by A and B and the rates of total gas flow by a, b, and c.

An equation giving x, the amount of radioactive gas in chamber I, as a function of t was derived in terms of the above constants.

The derivation is cumbersome and will not be presented here. The resulting solution,

$$X = \tfrac{1}{2}Ae^{-\alpha t/2}\left[\left(1 - \frac{\bar{\alpha}}{\sqrt{\alpha^2 - 4\beta}}\right)e^{\frac{1}{2}\sqrt{\alpha^2-4\beta}\,t} + \left(1 + \frac{\bar{\alpha}}{\sqrt{\alpha^2 - 4\beta}}\right)e^{-\frac{1}{2}\sqrt{\alpha^2-4\beta}\,t}\right], \qquad (2)$$

where

$$\alpha = \frac{a+b}{A} + \frac{b+c}{B}$$

$$\bar{\alpha} = \frac{a+b}{A} - \frac{b+c}{B}$$

$$\beta = \frac{a(b+c)}{AB},$$

gave graphs which were in some respects similar to plots of data obtained as described below. The model curves are shown in Fig. 4.

First B, the volume of chamber II, was set equal to A, the volume of I; and b, the exchange rate, set equal to a, the ventilation rate. On the left of the figure this curve is compared with one in which B is twice A with the other constants equal. On the right the same curve is compared with one in which b is half of a. From a study of these curves it is quite obvious that the shape of the curve is much more sensitive to the volume constants A and B (varied in the left-hand set of curves), than to the rate constants a and b (varied in the right-hand set).

Although the equation derived above is rigorous for the model it is far from rigorous for the experiments to be described. However, it does seem to approximate a certain phase of the dynamics of carbon dioxide metabolism.

Although the model describes a steady state in a gas chamber system, the same equations hold for any analogous system if a consistent set of units are employed. Chamber I can represent equally well a vessel of bicarbonate buffer through which CO_2 is diffusing, if the gas being measured in chamber I is $C^{14}O_2$. Actually, the model was designed to represent, at least as a first approximation, an experimental set-up in which an animal is exposed to $C^{14}O_2$. Chamber II was considered as the equivalent of an animal placed in chamber I which was ventilated at a constant rate (a) in order to keep the CO_2 concentration fairly level. "B" was thought of as that

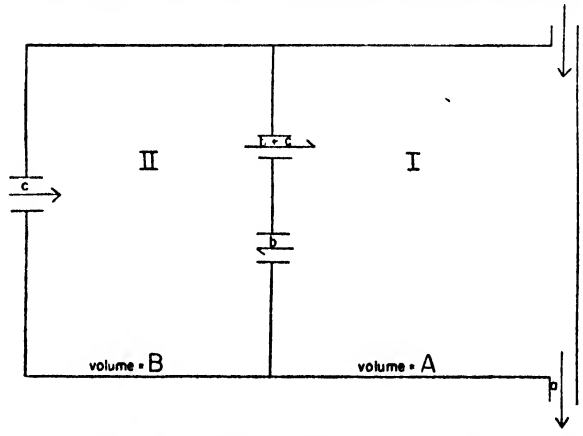

FIG. 3. Model of Flushed Chamber System (see text for explanation).

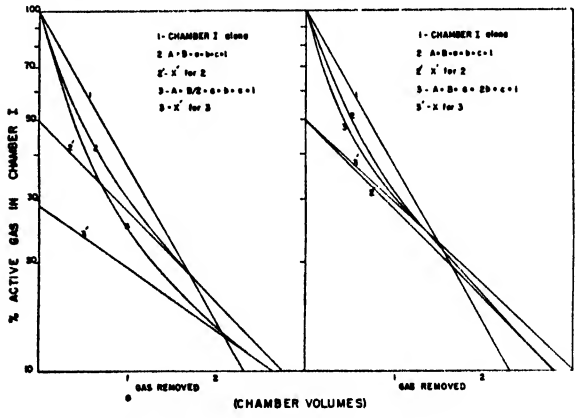

FIG. 4. Curves indicating the rate of dilution of $C^{14}O_2$ in the system shown in Fig. 3, varying both rate constants and chamber volumes. On the left, the chamber volume constants have been varied by a factor of two; on the right, the rate constants have been varied by the same factor.

pool of substances, inorganic and organic, which exchange C^{14} at a rapid rate with inspired $C^{14}O_2$, and (b) as the rate of exchange of radioactive CO_2 between the enclosing chamber (I) and this pool. The metabolic CO_2 was pictured in the model as entering the rapidly exchanging pool at a fairly constant rate (c). Experiments were conducted with this setup to determine whether the early portions of the curves so obtained resembled those of the mathematical model.

cc. vessels mounted five feet above a reservoir. The flow rate was adjusted so that each bulb emptied in slightly less than 12 minutes and since the bulbs were used alternatively each 12 minutes, a nearly continuous flow of gas from the chamber was maintained. While gas was entering one bulb from the chamber, the gas contents of the other bulb were replaced with acid from the reservoir below. This replacement was accomplished by suction on the gas which was bubbled through NaOH to trap the CO_2.

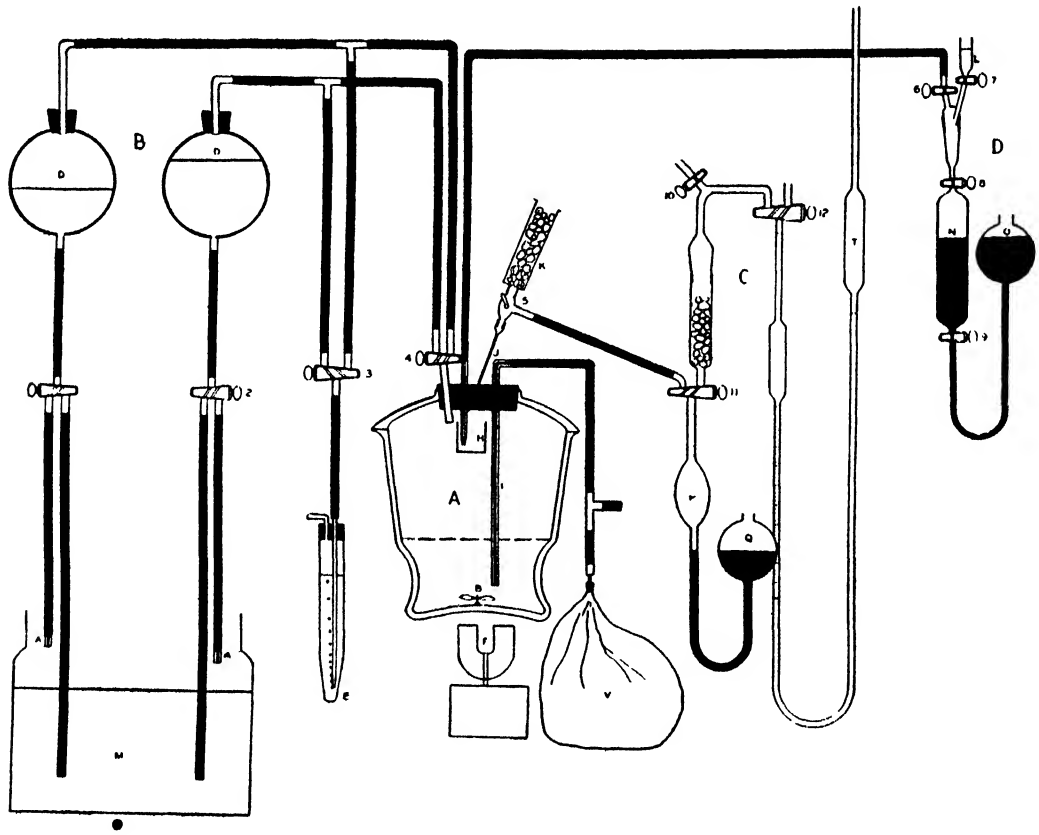

FIG. 5. Apparatus for flushed chamber experiments.

A, chamber (2480 cc.); *B*, sampling apparatus; *C*, CO_2 analyzer; *D*, CO_2 generator.

Experimental

The apparatus used in these experiments is diagrammed in Fig. 5. The chamber was a desiccator (volume, 2480 cc.) with a wire screen to support the animal. Mixing of gas in the chamber was insured by a small sheet metal fan which rotated in the field of an electrically driven horseshoe magnet mounted below the chamber.

Gas was removed from the chamber by means of gravity flow of dilute hydrochloride acid from 1120-

This alkali trap was changed with each 1120-cc. sample.

The gas removed from the main chamber in this manner was replaced by a flow of pure oxygen. A partially collapsed basketball bladder in the inlet line kept the chamber contents at atmospheric pressure. Back diffusion was minimized by the use of capillary tubing (2-mm.) bore).

The concentration of carbon dioxide in the chamber was measured at intervals by a simple volumetric

method. Ten cc. of gas were withdrawn from the chamber and then passed through soda lime. The resulting diminution in volume represents the amount of carbon dioxide in the sample. Water vapor corrections are unnecessary if the samples are saturated prior to analysis, as we assumed them to be in these experiments, inasmuch as vapor invariably condensed on the chamber wall. An analysis was performed every six minutes during each experiment.

$C^{14}O_2$ with the desired amount of carrier was generated and introduced into the chamber by means of apparatus D, shown in the diagram. A sample of pure dry barium carbonate containing about 250 μc. per gram was weighed and placed in the generator, which was then sealed. A partial vacuum was then produced inside the generator by lowering the mercury bulb. The gas formed on addition of acetic acid (20 cc. of about 30 percent) entered the main compartment of the apparatus where it remained at a reduced pressure until its introduction into the chamber.

Method

The experiments were performed as follows:

A rat was placed in the chamber and pure oxygen was rapidly drawn through for several minutes. The chamber inlet was clamped and suction continued for several seconds. This produced a slight negative pressure within the chamber which was then sealed by turning the outlet stopcock. The active gas was immediately introduced, the clamp removed from the inlet and the ventilation begun. About 30 seconds elapsed from the time that the inlet tube was clamped until the beginning of the first sample withdrawal.

During the experiment each sample (1120 cc.) was passed through a separate tube of 3N NaOH. Two cc. of $BaCl_2$ were added to each tube after the trapping and the tube was then stoppered and centrifuged. The precipitate was washed twice with CO_2 free water and twice with anhydrous methanol. After the final washing the precipitate was stirred into a paste and placed on flat aluminum dishes (¾ inch diameter). The samples were dried under a heat lamp and flattened with a modified Parr pellet press. All samples were in considerable excess of "infinite" thickness. The radioactivity was measured with a thin window Geiger-Mueller counter and compared with a standard prepared in triplicate from the starting $BaCO_3$.

After about six hours sampling by volume was discontinued. A large sintered trap was placed in series with the chamber after being filled with 3N NaOH. The oxygen inlet was replaced by a soda lime absorption tube. Ventilation with CO_2-free air at an accelerated rate was then continued by means of water suction. The trap was changed at varying intervals and the activity of the carbonate determined as described.

Before any animal experiments were performed with the apparatus, the emptying curve of the chamber alone was determined and compared with the curve obtained by plotting equation (1). This was done in two ways.

In the first method the chamber was filled with non-radioactive carbon dioxide and then ventilated with air by use of the hydraulic system as decribed. The carbon dioxide concentration was measured at intervals by means of the volumetric analyser.

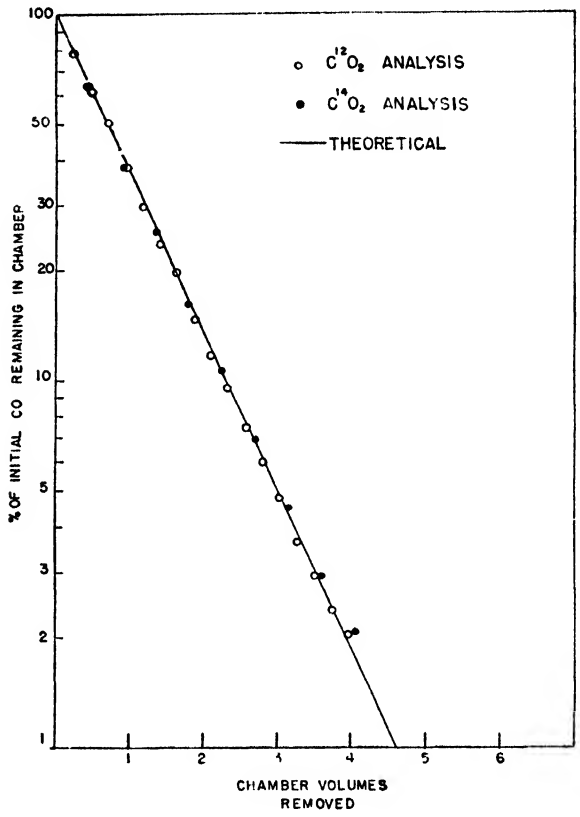

FIG. 6. Curve showing flushing rate of chamber used in experiments, with no rat in the chamber.

In the second method the chamber was filled with five percent non-radioactive CO_2 and then about 25 μc. of $C^{14}O_2$ were added. The inlet tube and the bag were connected to the tank of CO_2 and sampling begun as described. The samples were counted after preparation as described.

The results are shown in Fig. 6. The line is the curve given by equation (1) when the proper volume relationships are used. It can be seen that both sets of values follow this curve quite closely. It is believed that the slight deviation of the points obtained by the counting technique may be due to turnover of active gas with the dilute acid in the collecting system.

Analysis of data

The specific activity of each sample was calculated as percent of the specific activity of the initially introduced CO_2. These values were then converted to "absolute" activity values by multiplying the specific activity of each sample by the mean CO_2 concentration of the chamber during the interval of collection. The initial value was again set at 100 percent and all subsequent intervals adjusted accordingly.

The data were plotted as shown in Fig. 7. Each value was plotted as a mean over the interval of collection, and the curve so drawn that the mean of the curve over the interval was as close to the experimental value as a smooth curve would permit.

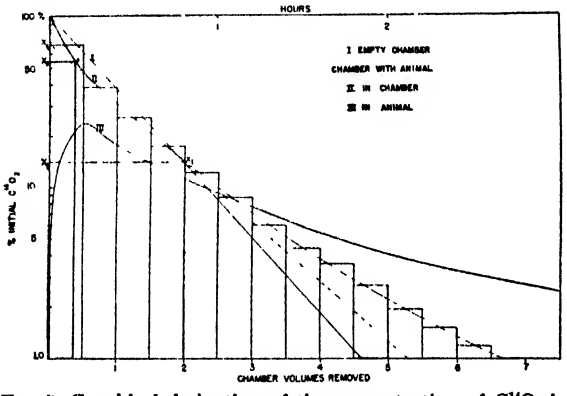

FIG. 7. Graphical derivation of the concentration of $C^{14}O_2$ in animal in a flushed chamber system, as a function of time.

The percent of total activity in the animal at any time may be calculated from the curve in the following manner. Since the amount in the chamber as a function of time is represented by the experimental curve, the integral of this curve from 0 to t is equal to the amount removed from the chamber in that time period. The percent of the total amount of $C^{14}O_2$ in the animal at any time is then equal to 100 minus the value of the curve at time t (the amount in the chamber) minus the integral of the curve from 0 to t. The units of t must be so chosen that a volume of gas equal to that of the chamber is removed in one unit of time. These computations result in a calculated curve similar to the lower curve in the figure.

Six experiments were performed as outlined above. Analysis of the data in these experiments failed to account for 100 percent of the activity introduced. The recovery ranged from 87 percent to 97 percent of the initial $C^{14}O_2$. Four of the experiments gave a recovery of greater than 95.2 percent. Analysis of the carcass of an animal which showed 11 percent "retention" yielded only .5 percent. Analysis of the acid in the gas generator for this same experiment accounted for an additional six percent, despite the fact that some of the liquid was lost in the tubing during the introducion of the gas.

It seemed likely that the losses were largely traceable to this source. Furthermore, the experiments in which the per cent recovery was the greatest were the ones in which the greatest amount of $BaCO_3$ was used. This fact supported the same hypothesis on a solubility basis.

The curves were corrected for this loss by subtracting the unaccounted-for activity from the initial value. The animal was considered to contain .5 percent in each case.

Data for one typical experiment are shown in Fig. 8. The experimental curve is compared with that of the empty chamber, and the calculated curves for the amount of C^{14} in the animal are also presented. It can be seen that the early part of the curve is qualitatively similar to that representing the mathematical model. However, the output curves are not exponential, as they are in the simpler system of the model. This, of course, is to be expected, since the model takes into consideration only a single rapid phase of carbon turnover. Therefore the observed deviation from the exponential rate seen in the model is a reflection of the "metabolic" phases of carbon metabolism, which cannot be neglected even in the short period of the experiment.

The early part of the curves seems to be almost linear, and this was especially true in the fasted animals. The values of "B" calculated from these

TABLE 2. EXPERIMENTS IN FLUSHED CHAMBER

	Weight of rat	Maximum uptake (% of total in chamber)	Maximum uptake %/gm.	Time of maximum uptake (min.)	Value of B expressed as mM./kilo	Mean CO_2 percent in chamber air	cc. air cleared per minute
Fischer rats, fasted	142.3	19.2	0.135	17	14.2	3.09	55.8
	151.5	20.0	0.132	12	14.5	3.30	76.4
Sprague-Dawley rats, fasted	263.0	28.0	0.106	10	17.2	5.83	150.5
	247.5	27.0	0.109	12	13.1	4.75	117.3
Fischer rats, not fasted	150.0	18.3	0.122	7	18.3	3.24	176.2
	159.0†	17.8	0.112	12	19.5	3.46	78.5

† Given 2.5 gm. glucose by stomach tube one-half hour before.

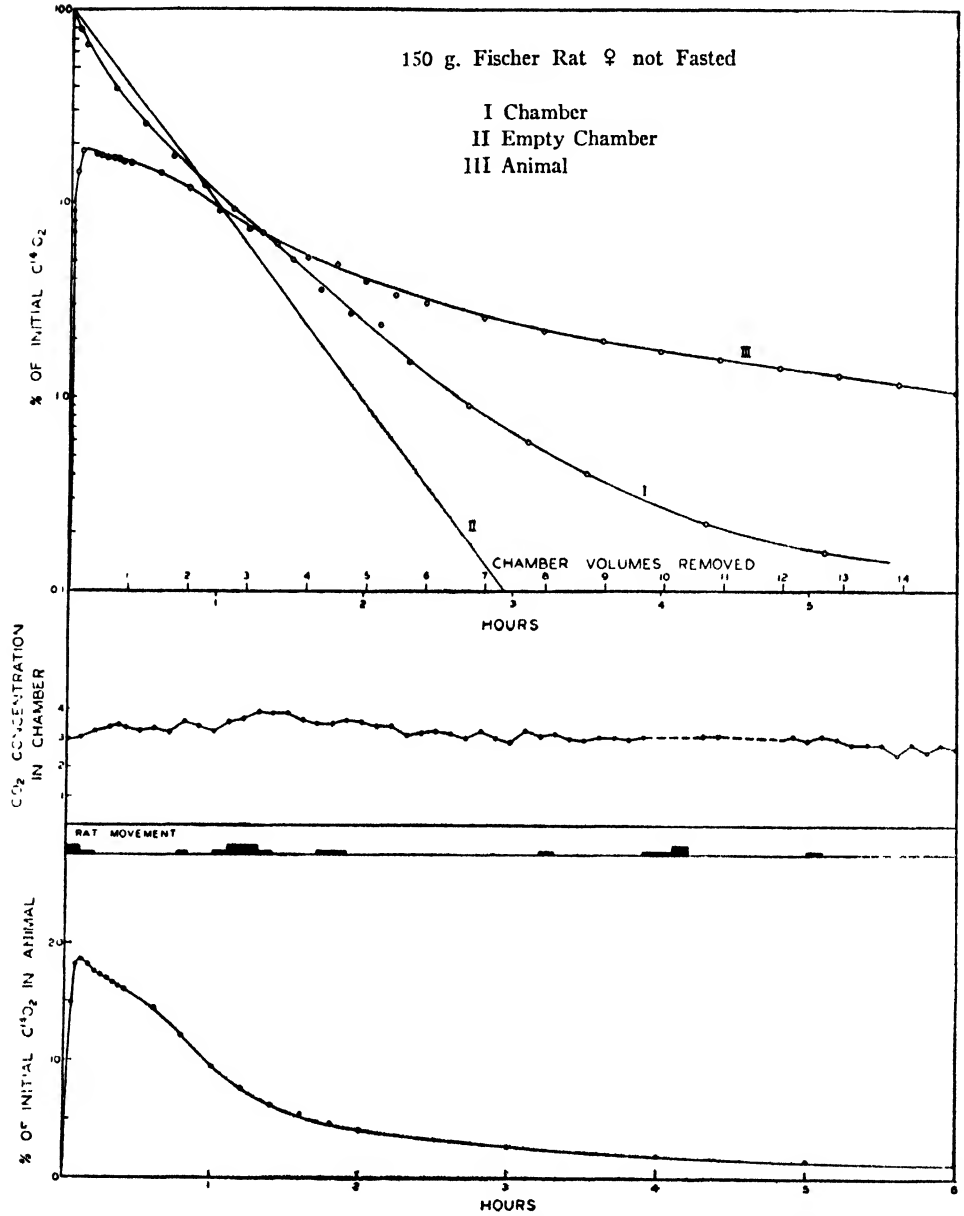

FIG. 8. Graph illustrating a typical experiment in the flushed chamber.

data according to the method diagrammed in Fig. 7 are shown in Table 2. The values so obtained are slightly higher than the estimated size of the total CO_2-bicarbonate system, but this difference is not significant because the animal presents a much more complicated system than does the model. From these values it would seem that there is no large pool of organic carbon in immediate exchange with the CO_2-bicarbonate system, and that the compounds which do exchange C^{14} with CO_2 or HCO_2^- in appreciable amounts do so at a somewhat slower turnover rate than that of the CO_2-bicarbonate system.

Table 2 also shows the maximum uptake of the labeled CO_2 for each animal as percent of the total activity originally introduced. These values for maximum uptake appear to be quite closely correlated with the surface area when the latter is estimated by the two-thirds power of the body weight.

Fig. 9 shows the combined data for all six experiments, including the terminal "washout" phases. The latter portions of the curves show that the net rate of excretion increases as the ventilation rate of the chamber is increased. The differences seen in the curves do not seem to be correlated with body

size or with the previous nutritional state of the animal. The upper curve is one obtained in a preliminary experiment in which an attempt was made to liberate the active CO_2 from wet $BaC^{14}O_3$ after the animal had been allowed to remain in the sealed chamber for a time sufficiently long to bring the CO_2 concentration to an anticipated steady state level. It was later found that the exchange of CO_2 with the $BaC^{14}O_2$ was appreciable, and that consequently during this period of about one hour the animal had been exposed to active CO_2 in an unventilated chamber.

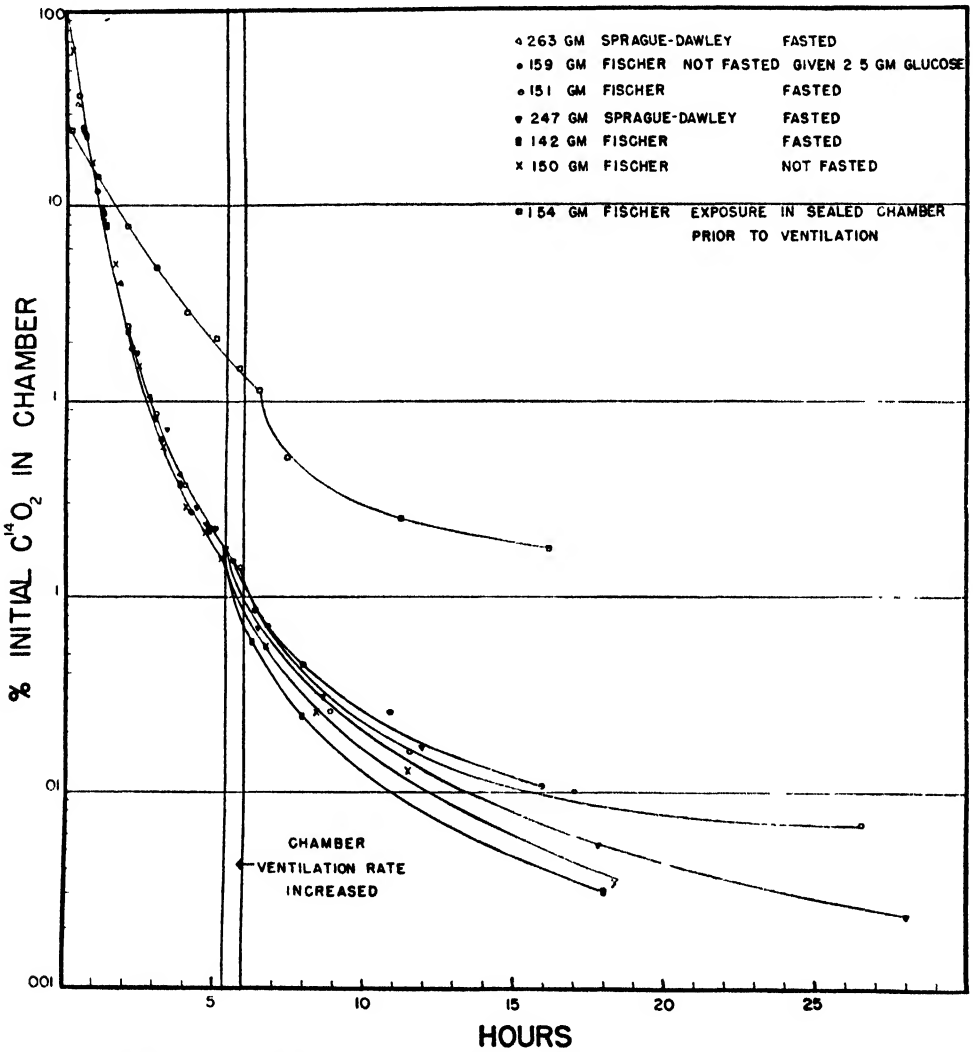

FIG. 9. Composite graphs of the series of flushed chamber experiments, showing chamber concentration of $C^{14}O_2$ as a function of time.

Retention in Growing Tissue

We have recently described (Brues and Naranjo, 1948) the uptake of C^{14} from bicarbonate by tissue cultures of chick embryo muscle, and its subsequent loss by degradation of the synthesized compounds. Cultivation of the tissue was done in a specially constructed bottle with a nylon window through which C^{14} of the tissue could be measured. After preliminary exposure to isotopic sodium bicarbonate for various periods of time, the cultures were washed with normal medium at 24-hour intervals for several days and incubated and allowed to grow between washings. This procedure was continued until loss of C^{14} proceeded at a very slow rate. As shown in Table 3, the degree of retention is a function of the duration of exposure and the rate of loss soon becomes very slow after a 48-hour exposure. Similar results have been obtained where tissues were explanted from chicks exposed during embryonic growth in the egg. The residual carbon appeared to

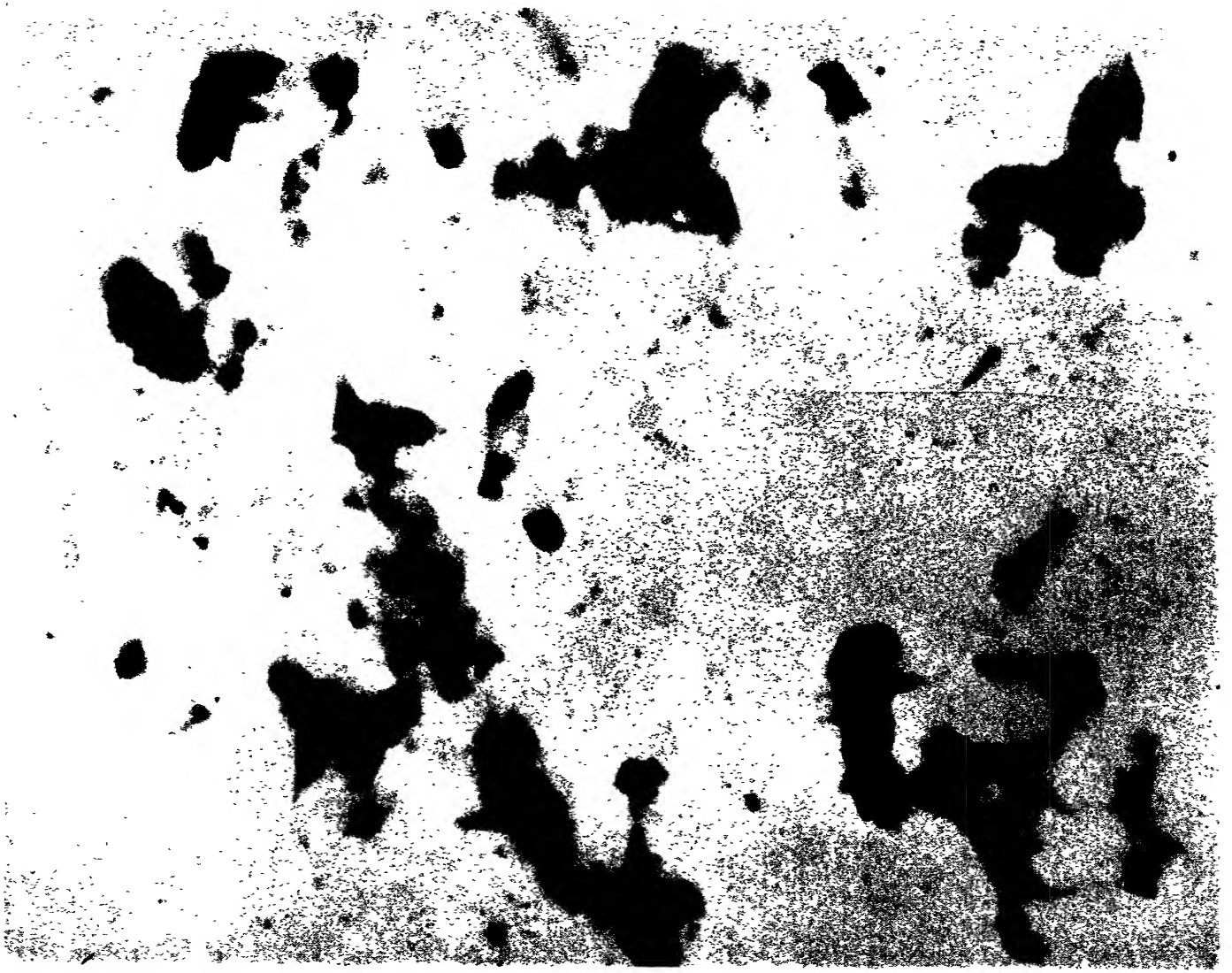

FIG. 10. Autoradiograph of chick embryo muscle explants, showing residual C^{14} in tissue after 48 hours' incubation followed by three 24-hour periods of incubation with C^{14}-free media. The tissue was washed with 5% trichloracetic acid, alcohol-ether, and saturated NaCl, and then autographed.

be in a variety of chemical forms, and about one-third was insoluble in dilute acid, fat solvents, and saturated NaCl (Fig. 10). Further studies on non-growing tissues will be necessary before the exact role of growth in favoring retention of fixed carbon can be evaluated.

Discussion

The preliminary information detailed here may be of some value in estimating the hazard of exposure to radioactive CO_2.

In the case of an exposure of limited duration, we see that (with the air renewed in about 20 minutes) the peak of uptake is passed within 20 minutes and does not exceed the amount initially in a gas phase two or three times the volume of the animal. At the end of six hours it approaches an amount corresponding to one-tenth the volume of the animal, and is still declining. If these conditions were true in the case of man, an initial exposure to a concentration of five microcuries per liter of air, although obviously to be avoided, would not result in storage of an amount in excess of a conservative tolerance amount after a few hours, if it occurred in a room with a comparable rate of air change. The slower ventilation rate relative to body mass in man would probably result in somewhat more favorable circumstances, since it seems clear that the ventilation rate is an important limiting factor in absorption.

Furthermore, since rapidly inhaled CO_2 can hardly be expected to be fixed in tissue to a greater degree than injected bicarbonate, it would appear from the data of Armstrong, Schubert and Lindenbaum (1948) that the fixation of 30 microcuries would require the inhalation of at least one hundred times that amount, if it were immediately followed by return to air free of radioactivity.

As to the removal of CO_2 from the blood after inhalation, it would appear that this might be accelerated by overbreathing, and this would be a practical measure following accidental inhalation. Clearance from the blood would probably also be facilitated by exercise, resulting in an increased pulmonary circulation and an increased net loss of carbon dioxide.

In the matter of chronic continuous exposure, we may make the extreme assumption that the specific activity of the blood carbonate system, and hence, eventually, of many carbon compounds in the body, will approach that of the alveolar air as a limit, which is approximately that of the external air concentration mixed with five percent CO_2. If we consider as a limiting case that a body concentration of five μc. per kilo of carbon (roughly 30 μc. in a man) is just to be avoided, this would be attained in equilibrium with alveolar air containing 360 liters of CO_2, corresponding to not over 7200 liters of room air per microcurie of $C^{14}O_2$ (5 disintegrations per second per liter, thus, being an absolute minimum).

If, as would be almost certain, the exposure were intermittent (*e.g.*, 8 hours out of 24) those compounds turning over very rapidly relative to an 8-hour period could be considered to be at "peak" specific activity one-third of the time, while those turning over relatively very slowly would gradually attain a maximum specific activity one-third of the "peak." Thus, the concentration required to produce a given integrated level of body radiation would be inversely proportional to the fraction of time during which exposure took place (in this case, by a factor of 3).

Table 3. Retention of C^{14} taken up from bicarbonate by cultures of chick embryo muscle over various periods of time, during the subsequent two days in non-radioactive media

Period of absorption (hours)	Percent retained by tissue after growth in non-radioactive media for:	
	24 hours	48 hours
48	39	33
24	19	16
6	10	6
2	7	3

In the case of growing tissue, the limiting case is represented by the assumption that all of the incorporated C^{14} is held permanently, after being laid down at the specific activity of the blood carbonate system. This would be alleviated, in the case of an exposure of short duration, by the incompleteness of synthesis during the exposure period, by the subsequent dilution of tissue radioactivity by further synthesis of non-radioactive compounds, and, of course, by degradation of the metabolizable components. With longer exposure, the circumstances would be bracketed by the case of chronic exposure already considered, in which the carbon concentration of tissue is assumed to be ten percent. This might be exceeded only in certain areas in bone. These matters are under further investigation.

Summary

Retention of C^{14} by fixation of inhaled $C^{14}O_2$ gas has been considered in the light of previously known facts and some preliminary experimental data.

Absorption of $C^{14}O_2$ by the lung occurs very efficiently, in spite of the mass movement of CO_2 in the opposite direction.

It is suggested that the amount absorbed to reach equilibrium with the blood bicarbonate system depends on the alveolar specific activity and thus, is largely independent of the CO_2 concentration of the inhaled air. A high external CO_2 concentration may increase the rate of absorption by inducing hyperventilation.

In general, following exposure, C^{14} is removed at a rate comparable to that of its uptake, and the same is probably true of its incorporation into compounds in a steady state. One probable exception exists in the case of growing tissue and, of course, where exposure is continuous over a long period. In these instances, it is estimated that five disintegrations per second per liter of air is the maximum amount which could safely be permitted. A few minutes exposure to over ten thousand times this concentration would probably not result in serious consequences.

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THEORETIC AND EXPERIMENTAL CONSIDERATIONS OF BIOLOGIC DECAY PERIODS: STUDIES IN MAN WITH THE USE OF Na^{22} [1]

G. E. BURCH,[2] S. A. THREEFOOT,[2] J. A. CRONVICH[3] AND P. REASER[2]

The rates of turnover of isotopes in organisms or isolated compartments of organisms are of considerable interest to general and human biologists. For obvious reasons, most studies of biologic decay of tracer materials have been conducted in small organisms or experimental animals. Through the kindness of Drs. A. L. Hughes and Martin Kamen of Washington University and Drs. M. Tuve and Dean Cowie of the Carnegie Institution in Washington, Na^{22} was obtained for certain experiments in man. Because of its long physical half-life (3 years), Na^{22} is the only isotope of sodium suitable for tracer studies which require relatively long periods of time. Although these studies were not designed primarily for observation of biologic decay periods in man, the data were suitable for analyses concerned with such problems both in normal man and in patients with chronic congestive heart failure or with the nephrotic syndrome of chronic glomerulonephritis. The rates of biologic decay were influenced by drugs and dietary factors studied in these experiments. Theoretic considerations, which are of some interest in tracer principles in general, were applied. It is the purpose of this presentation to summarize these data, which will be reported in more detail elsewhere (Burch, Threefoot and Reaser, 1948; Threefoot, Burch and Reaser, 1949; and Burch, Threefoot and Cronvich, 1949).

Materials and Methods

Twelve subjects were observed continuously for periods varying from 20 to 70 days. Four of these were normal, six had chronic congestive heart failure (2 slowly improving, 2 rapidly improving and 2 slowly becoming worse) and two had the nephrotic syndrome of chronic glomerulonephritis (see Table 1 for details).

Na^{22}, as NaCl in approximately 2 cc. of water, was administered intravenously to each subject. Doses of Na^{22} with an activity such that there were 17.7×10^6 disintegrations per minute (about 0.09 mc.) were administered to seven of the subjects, 12.5×10^6 (about 0.06 mc.) to three subjects, and 1×10^7 (about 0.05 mc.) to the other two subjects. The dosage was reduced as more sensitive counting equipment became available.

The urine was collected separately at each voiding, and at least daily blood samples were taken. The volume and radioactive count of each sample of urine were recorded so that the total elimination of radiosodium could be determined. Radioactive counts of blood serum were followed as an index of the concentration of radiosodium in the extracellular fluid.

Table 1. Clinical Data

Subject No.	Age in years	Sex	Wt., initial, in lbs.	Diagnosis
A. *Normal or control*				
1	41	M	142 5	Obliterative pleuritis
2	16	F	134	Acute rheumatic fever
3	33	F	121	Esophageal peptic ulcer
4	39	F	123	Duodenal ulcer
B. *Congestive heart failure* (slowly improving)				
5	47	F	153	Hypertension
6	48	F	162	Arterial hypertension
C. *Congestive heart failure* (rapidly improving)				
7	63	M	131.5	Hypertension
8	47	F	134	Rheumatic heart disease (inactive); auricular fibrillation
D. *Congestive heart failure* (slowly becoming worse)				
9	46	M	155.5	Syphilitic aortic insufficiency
10	54	F	129.5	Hypertension
E. *Chronic hemorrhagic nephritis* (nephrotic syndrome)				
11	15	F	138	Renal function 25–30% normal; slowly improving
12	28	F	285.75	Renal function 25–30% normal

The aliquots of serum and urine were delivered as free falling drops from a calibrated micropipette to the surface of discs of filter paper. When dry, the paper discs were cemented to metal discs so that the quantity and geometric characteristics of each sample remained constant. The preparations were counted for five minutes, and the necessary corrections for background were made. Data were recorded as counts per minute per cubic centimeter of

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fluid. For purposes of comparison, all counts were corrected to correspond to an injection of 17.7×10^6 disintegrations per minute for each subject.

Urinary excretion of Na^{22} was expressed in terms of percentage of injected Na^{22} not eliminated by the kidneys. This value, $\%N_t$, was calculated from the equation

$$\%N_t = \left[1 - \frac{\sum_{s=1}^{t} k_s}{N_0} \right] \times 100,$$

where

N_0 = injected Na^{22}, in counts per minute,

k_s = Na^{22} excreted only in the urine during the s^{th} day after injection, expressed in counts per minute.

$\%N_t$ = percentage of injected Na^{22} not excreted in urine by the end of the t^{th} day after injection.

Weights and fluid intake and output were recorded daily. The sodium intake was varied in some instances from *low* (1.7 gm. NaCl/day), to *regular* (8 gm. NaCl/day), to *high* (13.7 gm. NaCl/day), and the effect on the rate of excretion of radiosodium was noted. A mercurial diuretic[4] and other drugs frequently employed in the clinical management of congestive heart failure exerted some influence on the rate of excretion of the radiosodium in all subjects.

TABLE 2. THE INDIVIDUAL AND MEAN $C_{\frac{1}{2}}$ AND $U_{\frac{1}{2}}$ VALUES FOR THE SUBJECTS STUDIED

Subject No.	$C_{\frac{1}{2}}$	$U_{\frac{1}{2}}$	Days of continuous observation	Weight change, in lbs.
A. *Normal or control*				
1	14	30	62	− 3.5
2	13	9	22	−14
3	12	42	45	−11
4	14	34	65	2.25
Mean	13.3	28.8	48.5	− 6.6
B. *Congestive heart failure* (slowly improving)				
5	40	60	35	−18
6	42	72	46	− 7
Mean	41	66	40.5	−12.5
C. *Congestive heart failure* (rapidly improving)				
7	13	26	62	−29
8	28	33	58	−17
Mean	20.5	29.5	60	−23
D. *Congestive heart failure* (slowly becoming worse)				
9	24	72	68	17
10	30	48	58	− 5.5
Mean	27	60	63	5.75
E. *Chronic hemorrhagic nephritis* (slowly improving)				
11	58	660	45	15
12	54	366	71	−86
Mean	56	513	58	−35.5

RESULTS

Results are summarized in Table 2 and in Figs. 1, 2 and 3.

1. In the *control* subjects, who had no cardiovascular disease or edema, the serum concentration of Na^{22} decreased to half the initial level in an average of 13.3 days (Table 2 and Fig. 1). The rate of elimination of the isotope in the urine was such that one-half the Na^{22} administered would have been excreted in an average of 28.8 days. Normal subject No. 1 demonstrated the influence that intake of sodium chloride has upon the rate of elimination of Na^{22} (Fig. 1B); increase of the daily intake of NaCl from 1.7 gm. to 13.7 gm. resulted in a threefold increase in rate of decline of serum concentration of Na^{22}. Similar response was noted for rates of elimination in the urine (Fig. 3B).

2. The patients with *chronic congestive heart failure* responded differently from the controls, and their response was related in part to the state of failure.

(a) In two patients who were *slowly recovering* from heart failure, 40 and 42 days respectively were required for the serum concentration of Na^{22} to reach half the initial level (Table 2 and Fig. 2A)—approximately one-third the rate of the control subjects. The Na^{22} was excreted in the urine at a rate such that 60 and 72 days respectively would have been required for elimination of one-half the administered isotope (Table 2 and Fig. 3C)—essentially one-half the rate of the control subjects.

(b) The two patients who were *rapidly improving* required 13 and 28 days respectively for the serum concentration of Na^{22} to reach one-half the initial level (Table 2 and Fig. 2B). During the first few days of observation the rate of decrease in serum concentration of the isotope was relatively slow, but when recovery from the failure once began, the drop was rapid, and became more rapid in one patient than that observed in the control subjects. Rates of urinary excretion tended to parallel changes in concentration in the serum (Fig. 2B and 3D). These two patients eliminated Na^{22} more rapidly than the two who improved slowly.

(c) In two patients who *slowly became worse* there was a reduction of the serum concentration to one-half the initial level in an average of 24 and 30 days respectively (Table 2 and Fig. 2C). The mean rate of loss of Na^{22} in the urine was such that one-half the administered radiosodium would have been

[4] Mercuhydrin (sodium salt of methoxyoximercuripropylsuccinylurea with theophylline) furnished by courtesy of Lakeside Laboratories, Milwaukee.

excreted in 60 days (Table 2 and Fig. 3E). These patients required a longer period of time to excrete the Na^{22} than did the subjects discussed previously.

3. The two patients with the *nephrotic syndrome of chronic glomerulonephritis* had the slowest rates of Na^{22} excretion. Decrease in the serum concentration of Na^{22} was such that an average of 56 days would have been required to reach one-half the initial level (Table 2 and Fig. 2D). The rate of urinary excretion was also extremely slow in both patients; an average of 513 days would have been necessary to eliminate one-half the Na^{22} administered (Table 2 and Fig. 3F).

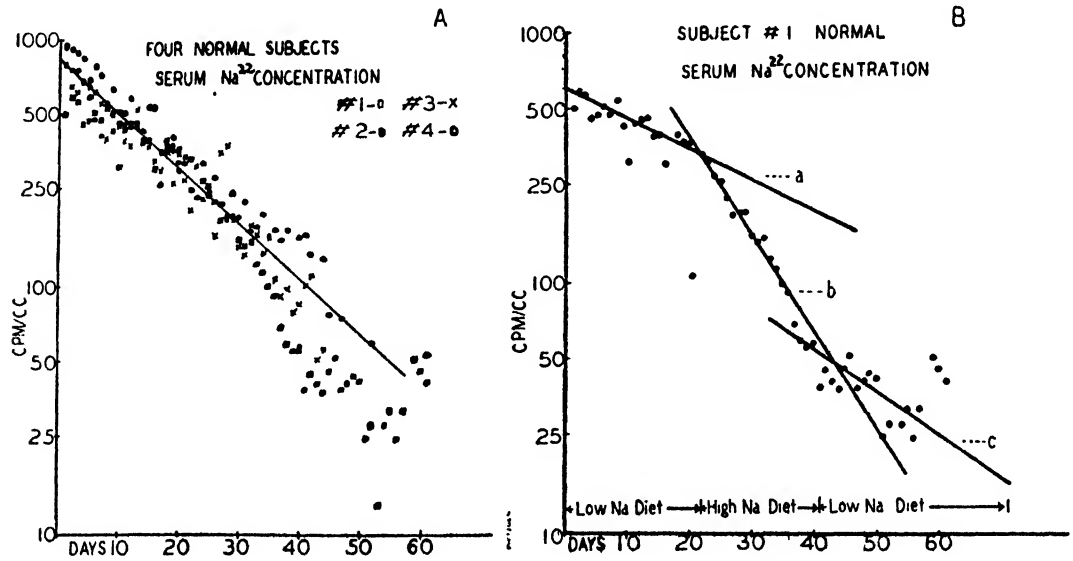

FIG. 1. Semilogarithmic graphs of relation of changes in serum concentration of Na^{22} (counts per minute per cubic centimeter) to time.

A. *Four subjects without cardiovascular disease.* The mean rate of fall in concentration was such that half-concentration was reached in 13.3 days ($C_{1/2}$).

B. *Normal subject No. 1* shows a change in rate of fall in serum concentration of Na^{22} with variations in sodium content of the diet, i.e. low sodium diet (1.7 gm. NaCl daily) and high sodium diet (13.7 gm. NaCl daily). At rate *a*, with low sodium diet, serum concentration reached half the initial value in 25 days. At rate *b*, with a high sodium diet, half serum concentration was reached in 8 days, and at rate *c*, when a low sodium diet was resumed, half-concentration was reached in 18 days.

COMMENT

Morgan (1947) suggested the symbol "Te" for "the body elimination half-life"; this term might be satisfactory if the time required to eliminate one-half the radioactive material administered could be determined without too much difficulty. Unfortunately, this is not always easily accomplished in man, especially for sodium. Results showed considerable variations in man, influenced especially by disease, drugs, diet, previous physiologic state and many other factors. It is therefore possible to determine only approximately the time required to eliminate one-half the administered isotope. Because values obtained for sodium differed from those found for the urine, it was necessary to introduce new terms:

$B_{1/2}$ = *biologic half-life,* the time required to eliminate one-half the administered tracer substance from the body. This corresponds to the "Te" value of Morgan (1947).

$C_{1/2}$ = *concentration one-half,* the time required for the concentration of the tracer material in the body fluid or substance or specific compartment to reach one-half the concentration existing at any time after equilibrium of distribution of the substance has been reached. It is thus possible to consider $C_{1/2}$ for the cerebrospinal fluid, $C_{1/2}$ for hepatic parenchyma, $C_{1/2}$ for blood serum.

$U_{1/2}$ = *urinary elimination one-half,* the time required to eliminate in the urine one-half of the tracer substance administered.

From the point of view of calculating safe dosages (Morgan, 1947), it is the $C_{1/2}$ that is important. However, $C_{1/2}$ measurements may not indicate rates of turnover or elimination from the body if the compartment for the tracer changes in size. Moreover, $C_{1/2}$ and $U_{1/2}$ will differ, since they are usually concerned with physiologic phenomena which are similar only under certain conditions. This problem will be discussed more fully later.

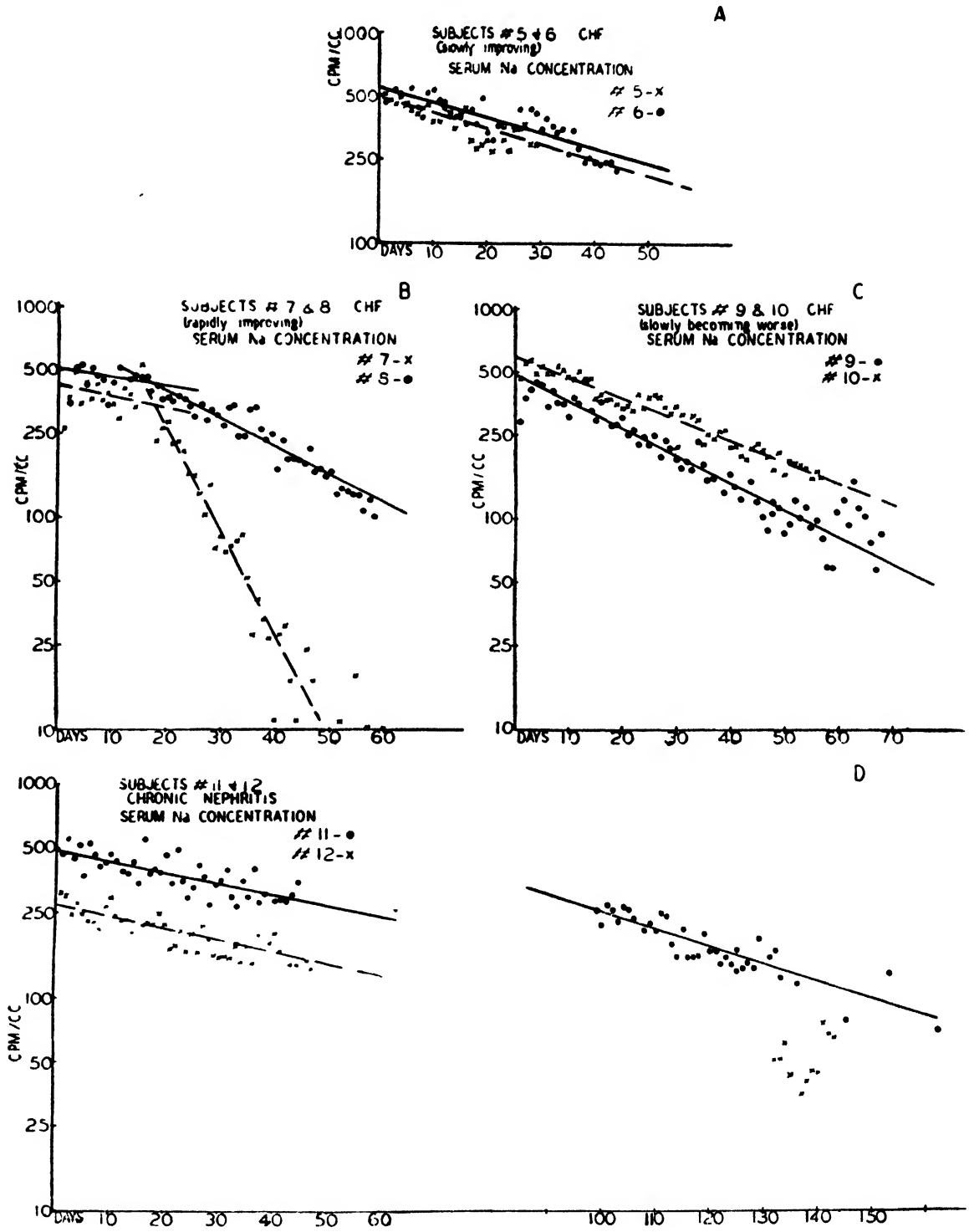

FIG. 2. Semilogarithmic graphs of changes in serum concentration of Na^{22} (counts per minute per cubic centimeter) as a function of the time for 6 patients with congestive heart failure and for 2 patients with the nephrotic syndrome of chronic glomerulonephritis.

A. *Two patients with congestive heart failure who were slowly improving. Patient No. 5* showed a mean rate of fall in serum concentration of Na^{22} such that half-concentration was reached in 40 days ($C_{1/2}$). For *Patient No. 6*, 42 days were

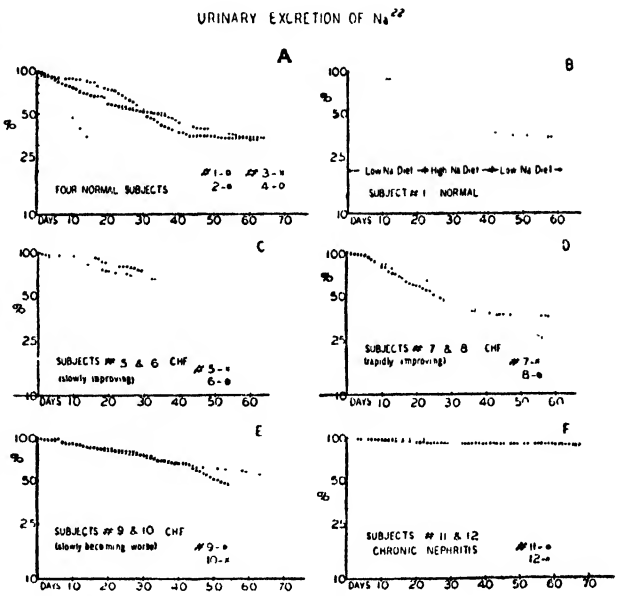

FIG. 3. Semilogarithmic graphs for all subjects showing the urinary excretion of Na^{22} (percentage of injected Na^{22} which was not eliminated by the urine). This value was obtained from the total counts of Na^{22} excreted daily through the urine. That for the first day was subtracted from the total counts injected; the total for each successive day was subtracted serially. Each resultant was then expressed as a percentage of the total injected dose. These values represent that radiosodium which remained within the body plus that which was excreted by some other route, i.e., that Na^{22} which had not been eliminated in the urine. This also indicates the *rate* of excretion by way of the urine.

A. *Four subjects without cardiovascular disease.* If all the Na^{22} were eliminated in the urine, 28.8 days (mean value, $U_{1/2}$) would be required for the injected radiosodium to be excreted.

B. *Normal subject No. 1* shows changes in rate of excretion of Na^{22} in the urine with changes in the sodium content of the diet. For 22 days during a low sodium diet the rate of elimination in the urine was such that one-half the sodium present in the body would have been eliminated in 100 days ($U_{1/2}$). For the next 19 days during a high sodium diet the rate of excretion increased, so that one-half the body sodium would have been excreted in the urine in 19 days. For the last 19 days of observation during a low sodium diet and administration of antidiuretics, the rate was such that 250 days would have been required for excretion of one-half the sodium present at the beginning of that period. Actually one-half the injected Na^{22} was excreted in 30 days.

C. *Two patients with congestive heart failure who were slowly improving.* Several different rates of excretion for each patient may be noted, the mean rate for the 2 patients being 66 days for excretion of half the Na^{22} injected.

D. *Two patients with congestive heart failure who were rapidly improving.* Several rates of excretion may be observed. The mean length of time required to excrete one-half the injected Na^{22} by the urine was 29.5 days ($U_{1/2}$).

E. *Two patients with congestive heart failure who were slowly becoming worse.* Changes in rate of excretion may be noted as in the other subjects. The mean time necessary for excretion of one-half the injected sodium through the urine was 60 days.

F. *Two patients with the nephrotic syndrome of chronic glomerulonephritis.* If sodium were excreted only by the urine, a mean of 513 days would have been required to excrete one-half the injected radiosodium.

THEORETIC CONSIDERATIONS

More careful consideration of the $C_{1/2}$ data presented reveals the numerous difficulties which arise when an attempt is made to compare the values for the control subjects with those for the edematous patients. Certain factors are worthy of discussion. For example, the excretion of an isotope is related to the following phenomena:

1. There is daily reduction in concentration and/or total amount of the isotope within the body because of continuous excretion. Experimental results indicate that excretion is exponential in character.

2. Change in concentration of the tracer material results whenever the volume of the compartment of the tracer varies.

LEGEND FOR FIGURE 2—*continued*

required. It may be noted on the graphs that for each of these patients several rates of change existed, although only the mean rate is shown by the straight line.

B. *Two patients with congestive heart failure who were rapidly improving. Patient No. 7* showed two distinct rates of fall in serum Na^{22} concentration. The first rate maintained for 18 days, was such that half-concentration would have been reached in 6 days. *Patient No. 8* also showed two distinct rates of fall; the first, present for 18 days, was such that half-concentration would have been reached in 71 days, whereas with the second rate 24 days would have been necessary.

C. *Two patients with congestive heart failure who were slowly becoming worse.* For *Patient No. 9* the mean rate of fall in serum concentration of Na^{22} was such that half-concentration was reached in 24 days ($C_{1/2}$), and for *Patient No. 10* in 30 days. Several rates of change in concentration may be noted, although only the mean rate for each patient is indicated by the straight line.

D. *Two patients with the nephrotic syndrome of chronic glomerulonephritis.* Both of these patients were discharged from the hospital and later readmitted for continuation of the studies. On the first admission *Patient No. 11* showed a mean rate of fall in serum concentration of Na^{22} so that half-concentration would have been reached in 58 days ($C_{1/2}$). During the second period of study this patient showed a mean rate of fall in concentration so that half-concentration would have been reached in 37 days, and *Patient No. 12* required 54 days.

3. Daily intake and elimination of the non-tracer form of the substance affects the behavior of the tracer form.

Although many other factors are concerned with the elimination of the tracer, only the foregoing three will be discussed since they deserve constant attention during experimentation. Any one of these may alter appreciably the concentration and rate of elimination of the tracer substance independent of the physicochemical process under observation.

It is advisable to define certain terms employed.

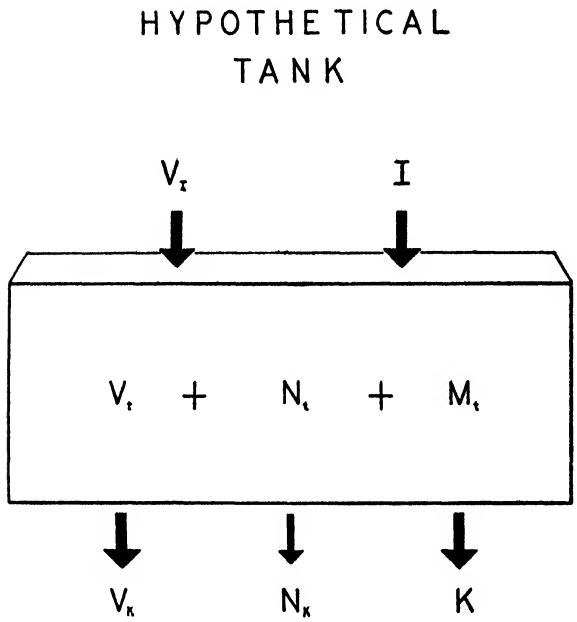

FIG. 4. Diagram of a "tank" or compartment containing Na^{23} in water in which the concentration of Na^{23} remains constant. Na^{22} as a tracer substance is added at an instant to "label" the Na^{23}. The Na^{23} and Na^{23} are always thoroughly mixed. To this tank more Na^{23} and water are being added continuously while Na^{22}, Na^{23} and water are constantly being removed. The problem is to determine the change in total quantity and concentration of the Na^{22} (the tracer substance) within the tank for various rates of intake and output of Na^{23} (the non-tracer substance). Man, or any organism or portion of an organism, may be compared to the tank. See text for details.

Elimination denotes any movement of the substance being studied from the compartment under observation.

Intake refers to any addition of the non-tracer substance to the compartment under study.

Volume of the compartment indicates the volume or space of that portion of the organism under study with the tracer. It is much more satisfactory to include in a compartment that portion of the organism which is physiologically (physically and chemically) homogenous. When a tracer, such as sodium, has several compartments with different rates of turnover, the problem becomes complex, especially if there are sudden changes.

With the use of these three terms, it is possible to apply the ideas presented hereafter to the organism as a whole or to any portion of the organism, even as small as a chromosome or gene.

The *problem* under consideration can best be approached through the aid of Fig. 4, which represents a tank. At time t this tank contains a compartment with a volume V_t, into which is dispersed a substance M_t, in solution or other state, to be observed. To this tank there is continuously being added more of the material which constitutes the dispersing compartment or medium, V_t, as well as more of the substance to be observed, I. Simultaneously, a certain amount of the dispersing medium V_K, and a certain amount of the substance under observation, K, are being removed continuously from the tank. If it is desired to trace this substance within the tank, then at a time $t = 0$ the known quantity of the tracer form, N, should be added to the tank. It is important to note that only a single addition of the tracer substance is made at one instant and that it rapidly becomes thoroughly mixed with the non-tracer.

1. If CN, the concentration of the tracer substance, is being observed in order to gain quantitative and/or qualitative information concerning the tracer substance, N, and the non-tracer form, M, then:
   (a) CN changes if there is escape of N from the tank and V does not change proportionally.
   (b) CN changes if N_K (the excretion rate of N) is zero, but V changes.
   (c) CN is dependent upon the relative variations in N_K and V. V, of course, depends upon the relative variations in V_I and V_K.

2. The total quantity of N within the tank at any given time varies with the rate of discharge of N from the tank.

3. The tank may be compared with any living organism such as man. Under biologic conditions further restrictions, such as isotonicity, must be imposed. For example, if the osmotic force of M within V is constant, then the variations in concentration of N and M and in the quantity of N, M and V will be determined by the relative intake and elimination of M and V.

When an analogy *between the tank and an organism* or any part of an organism is drawn, it is necessary to consider such factors as state of equilibrium, homogeneity of the compartment and substances under study, functional disturbances related to the problem and physiologic, physical and chemical peculiarities inherent in the substance being traced and in the compartment in which it resides. These and other matters will become evident as the discussion progresses.

It is possible to compare man with the tank in which M is sodium (Na^{23}), V is the sodium com-

partment, N is the tracer sodium (Na^{22}), I is the daily intake of Na^{23} in the diet, V_I is the daily intake of water, K, N_K and V_K are respectively the amounts of Na^{23}, Na^{22} and water eliminated daily. Isotonicity of the solution of M (Na^{23}) must be maintained. Therefore, CN (concentration of Na^{22} in the tank) will vary with the relative intake and output of V and, of course, with the relative values of I and K. In general, it is possible to disregard the mechanisms of metabolic processes within a compartment concerned with the sodium turnover when the compartment as a whole is considered.

For a more thorough understanding of the quantitative and qualitative nature of some of the important factors, unrelated to purely metabolic processes, which influence concentrations and total quantity of the tracer substance, certain interesting and essential equations were derived.

Mathematical Considerations

Another publication (Burch, Threefoot and Cronvich, 1949) contains a detailed discussion of the mathematical considerations. If a whole organism is considered as a tank, the mathematical theories outlined below will hold, regardless of the complex metabolic processes within the organism.

The equations may be applied to any types of tracer studies which satisfy the conditions imposed by the following essential assumptions. That these assumptions are reasonable is shown by the agreement of the theoretic and experimental data compared later in the presentation.

1. Tracer and non-tracer substances are uniformly mixed in the organism and are affected similarly by chemical and physical processes in the organism.
2. All of the tracer substance is added at time t = 0 and is rapidly mixed completely with the non-tracer substance.
3. Intake and discharge are continuous processes.
4. The changes in the quantity of the non-tracer substance and in the volume of its compartment vary exponentially. This is a physiologic variation which has been observed in our studies.
5. The solution of certain non-tracer substances, such as sodium, is assumed to be isotonic.

Three conditions were then analyzed (Burch, Threefoot and Cronvich, 1949). The equations for each are as follows:

Symbols

M_t = time
t = quantity of non-tracer substance in organism at time t
M_o = quantity of non-tracer substance in organism initially
I = quantity of non-tracer substance taken into organism per unit time
G = net quantity of non-tracer substance gained by organism per unit time (negative G = loss)
K = quantity of non-tracer substance eliminated from organism per unit time
D = total amount of non-tracer substance when quantity in organism is increasing
a = fraction of non-tracer substance in organism eliminated from organism at any time t—an expression of the rate of elimination
α = fraction of difference between maximum of the non-tracer substance to be reached and amount present at time t—an expression of the rate of accumulation
N_t = quantity of tracer substance in organism at time t
N_o = quantity of tracer substance in organism initially
V_t = volume of compartment under study at time t
V_o = initial volume of compartment under study
CM = concentration of non-tracer substance in organism
CN_t = concentration of tracer substance in organism at time t
CN_o = concentration of tracer substance in organism initially

Condition 1: Where G is negative or organism or tank is in a negative balance for Na^{23}

$$N_t = N_0 \mathcal{E}^{I(1-e^{at})/aM_0} \mathcal{E}^{-at} \tag{1}$$

$$CN_t = CN_0 \mathcal{E}^{I(1-e^{at})/aM_0}. \tag{2}$$

Condition 2: Where G is positive or organism or tank is in a positive balance for Na^{23}.

$$N_t = N_0 [M_0^{-1+I/(M_0+D)\alpha}] \cdot [M_0 + D(1-\mathcal{E}^{-\alpha t})]^{1-I/(M_0+D)\alpha} \cdot \mathcal{E}^{-It/M_0+D)} \tag{3}$$

$$CN_t = CN_0 [M_0^{I/(M_0+D)\alpha}] \cdot [M_0 + D(1-\mathcal{E}^{-\alpha t})]^{-I/(M_0+D)\alpha} \cdot \mathcal{E}^{-It/(M_0+D)} \tag{4}$$

Condition 3: Where $G=0$, or the intake of non-tracer substance is equal to the output for Na^{23}.

$$N_t = N_0 \mathcal{E}^{-It/M_0} \tag{5}$$

$$CN_t = CN_0 \mathcal{E}^{-It/M_0} \tag{6}$$

Note: $\mathcal{E}$ = Base of natural log.

The equations were applied to certain theoretic but clinically compatible situations concerned with the study of sodium following a single injection of Na^{22} in man with and without chronic congestive heart failure and generalized edema.

Application 1.—A man with generalized edema such that his total Na^{23} mass is 121.4 grams and ex-

tracellular fluid 34,000 grams, his edema is progressively disappearing, and he finally reaches the sodium (Na^{23}) and extracellular fluid mass for a 70 kilogram man (50 and 14,000 grams respectively).

For a better understanding of the influence of the rates of intake and output of the non-tracer upon the concentration and total content of the tracer, the rate of intake of the non-tracer substance and the rate with which the subject became edema-free were varied. Fig. 5 shows the theoretic progressive

large total elimination of Na^{22}. This is to be expected, since water is being lost simultaneously at a rate which maintains isotonicity of the Na^{23}.

(b) Differences in the rates of elimination of Na^{23} from the subject are accompanied by less change in the concentration of the Na^{22} in the extracellular fluid than in total quantity of Na^{22} present within those fluids. Similarly, this is reasonable because water is being eliminated with the Na^{23} to maintain isotonicity.

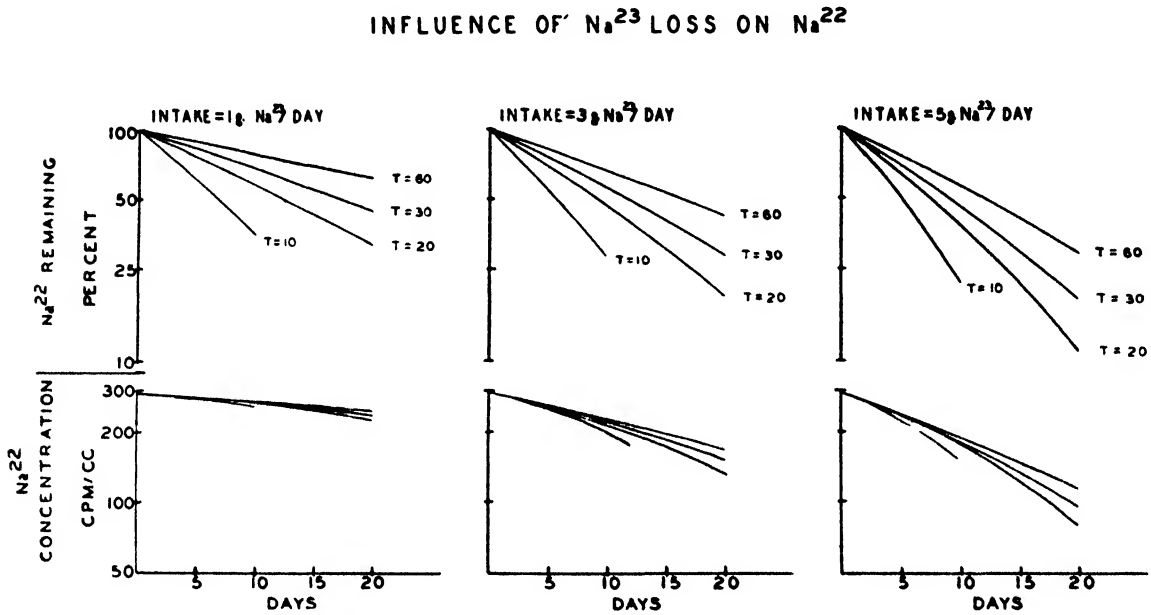

FIG. 5. Semilogarithmic graph showing the influence of the net loss of Na^{23} and the Na^{23} content of the "body" after administration of a single dose of the tracer. The influence of the rate of Na^{23} intake upon the Na^{22} is indicated. "T" denotes the time in days required for an *edematous individual* with a total of 121.4 grams of Na^{23} in the body to become edema-free and for the Na^{23} content to be reduced to 50 grams. The upper family of curves shows the time variations in the percentage of the initial Na^{22} remaining in the body, and the lower family of curves indicates the time variations in concentration of the Na^{22}. The T values follow the same order for the lower family of curves as indicated for the upper ones. For the curve showing rapid rate of Na^{23} loss or rapid rate of elimination of edema ($T = 10$ days), the patient reached the edema-free state in 10 days and the curve therefore does not extend as far on the abscissa as those for the other rates. It is interesting to note that the curves are *not straight lines*. For convenience the other curves were not continued beyond 20 days; if they were extended until the subject became edema-free, the amount of Na^{23} remaining in the body would be progressively less as the value of T increased.

change in total content and concentration in the extracellular fluid of Na^{22}, the tracer, for rates of excretion which would make the subject free of edema in 10, 20, 30 and 60 days when the daily intake of the non-tracer (Na^{23}) was 1, 3 or 5 grams.

This figure shows:

(a) When the intake of Na^{23} remains constant, its rate of elimination so influences the Na^{22} in the extracellular fluid that determination of the concentration of Na^{22} in the blood serum alone cannot serve as an index of the amount of Na^{22} still remaining in the body; a small decrease in the concentration of Na^{22} is associated with a relatively

(c) The rate of intake of Na^{23} has a greater influence upon the change in concentration of Na^{22} in the extracellular fluid than does the rate of elimination of the edema (Fig. 6). A greater intake of Na^{23} results in a more rapid rate of elimination of Na^{22} as well as in a sharper rate of decline in concentration of Na^{22} in the extracellular fluids. This effect of Na^{23} intake upon Na^{22} must exist, because the rate of elimination of the former must increase in order to produce the edema-free state at the given time. Since the movement of Na^{22} is determined by the movement of Na^{23}, then a greater intake with resulting increased elimination of Na^{23},

effects an increased rate of elimination of Na^{22}.

(d) Elimination of Na^{22} under the circumstances described is not a simple exponential phenomenon.

(e) It is evident from the equations and from Fig. 5 and Fig. 6 that when a subject is losing edema, the rate of excretion of Na^{23} greatly influences the Na^{22} content of the body, since the volume of the extracellular fluid decreases at a rate which insures isotonicity. The rate of intake of Na^{23} is important because of its effect upon its own rate of elimination.

(f) When a subject is progressively excreting the non-tracer substance under the conditions defined, the rate of change in *total amount* of the tracer substance retained is greater than the rate of change in *concentration* of the tracer. This is true because the volume of the compartment of the tracer and non-tracer substances is diminishing progressively as the Na^{22} is being eliminated.

(g) The concentration of Na^{23} in the extracellular fluids is constant, a necessary condition since isotonicity is essential for life. Concentration of Na^{22}, however, is not constant, because Na^{22} is

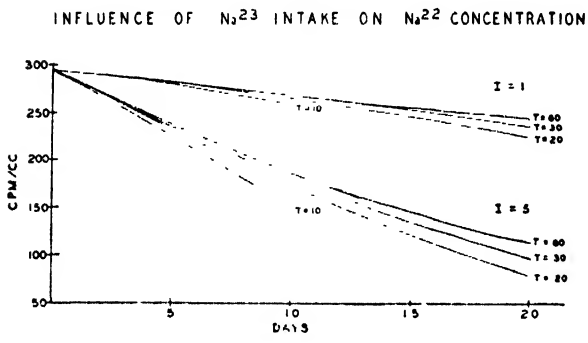

FIG. 6. The concentration curves of Fig. 5 drawn on ordinary cartesian coordinate paper for the intakes of 1 gram and 5 grams daily respectively. Note the greater change in concentration when the intake of Na^{23} is increased.

gradually being eliminated from the body without any associated continuous addition.

Application 2.—A man free of edema and weighing 70 kilograms, whose total sodium (Na^{23}) and extracellular fluid masses are 50 and 14,000 grams respectively and in whom edema is progressively developing until his sodium and extracellular fluid masses reach 121.4 and 34,000 grams respectively.

Figure 7 summarizes the theoretic changes in concentration and total content of the tracer (Na^{22}) in the extracellular fluids as influenced by various rates of development of the edematous state and by rates of intake of the non-tracer (Na^{23}). The calculations from which these curves were drawn yielded the following facts:

(a) A subject cannot acquire edema and accumulate sodium at a rate faster than the intake, except where special consideration must be given to storage depots and local shifts.

(b) When Na^{23} is being accumulated during progressive formation of edema, the rate of decrease in concentration of Na^{22} exceeds the rate of change in total content of the tracer within the body. This is to be expected, since the concentration of Na^{22}

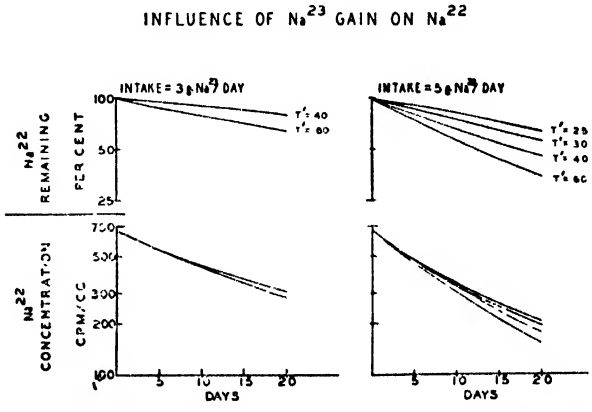

FIG. 7. Semilogarithmic graph showing the influence of the *net gain* of Na^{23} on the Na^{22} content of the "body" after administration of a single dose of the tracer. The influence of the rate of Na^{23} intake upon the Na^{22} is indicated. T′ denotes the time in days required for the total sodium of a *non-edematous* individual to increase from 50 grams to 121.4 grams, when an arbitrary maximum of edema is reached. The labeling scheme is the same as in Fig. 5. Each curve is not a straight line, for obvious reasons. Values of T′ smaller than those shown could not be analyzed under the conditions of the calculations (see text). Note the inverse relationship in the order on the ordinate positions of the curves for the respective T and T′ values of this figure and that of Fig. 5.

is being reduced because of *two* factors acting simultaneously:

(1) Accumulation of the fluid of edema, which produces a dilution of the Na^{22} and

(2) Continuous elimination of Na^{22}.

Reduction in the total content of Na^{22} within the body results from excretion only.

(c) Intake of Na^{23}, and of course its output, has a greater influence on the concentration and total content of Na^{22} within the body than does the rate of development of the edema.

Application 3.—A man whose electrolyte and water balances are stationary.

In this situation, in which the quantity of Na^{23} in the body remains constant and in which the extracellular fluid remains isotonic, the rate of elimination of Na^{23} becomes all important. The rate of intake exerts its influence upon excretion; when G is equal to zero, then intake and output must be equal.

Results of the calculations, shown in Fig. 8, indicate that:

(a) The greater the elimination and of course the intake of Na^{23}, the greater the rate of elimination of Na^{22}. This is tenable because the movement of the non-tracer substance governs the movement of the tracer.

(b) When G is equal to zero, the influence of intake and output on the concentration of the tracer is identical with that in the total content.

(c) When G is equal to zero and the tracer substance is not fixed in the organism in any manner, $B_{1/2}$ and $C_{1/2}$ are equal. These parameters are equal to $U_{1/2}$ if the elimination is entirely in the urine.

(d) When G is equal to zero, the decay curves for concentration and for total quantity of the tracer remaining in the organism are simple exponential curves which are straight parallel lines when plotted on semilogarithmic paper.

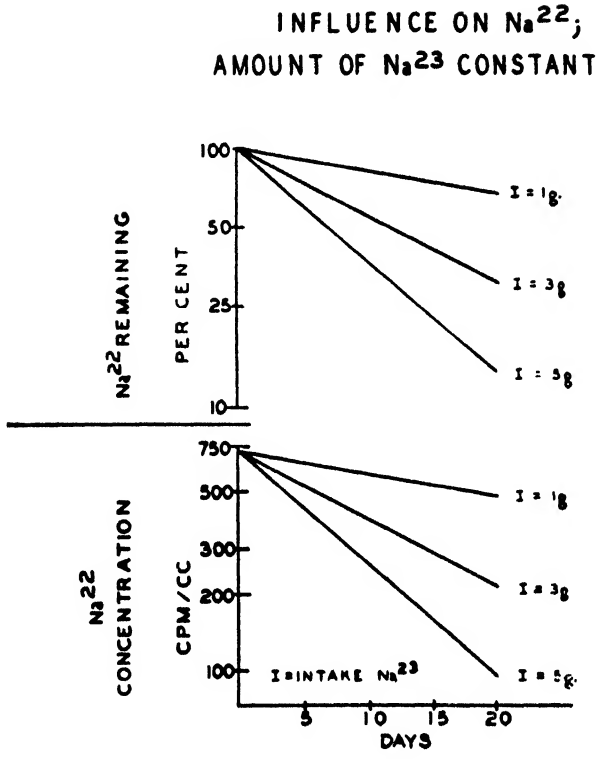

FIG. 8. Semilogarithmic graph showing the influence of *no change in the total* Na^{23} upon the Na^{22} content of the "body" after administration of a single dose of the tracer. Although the total amount of Na^{23} does not change, Na^{23} is being taken into the body and also being eliminated at rates of 1, 3 and 5 grams daily. These curves are all *straight lines,* for obvious reasons.

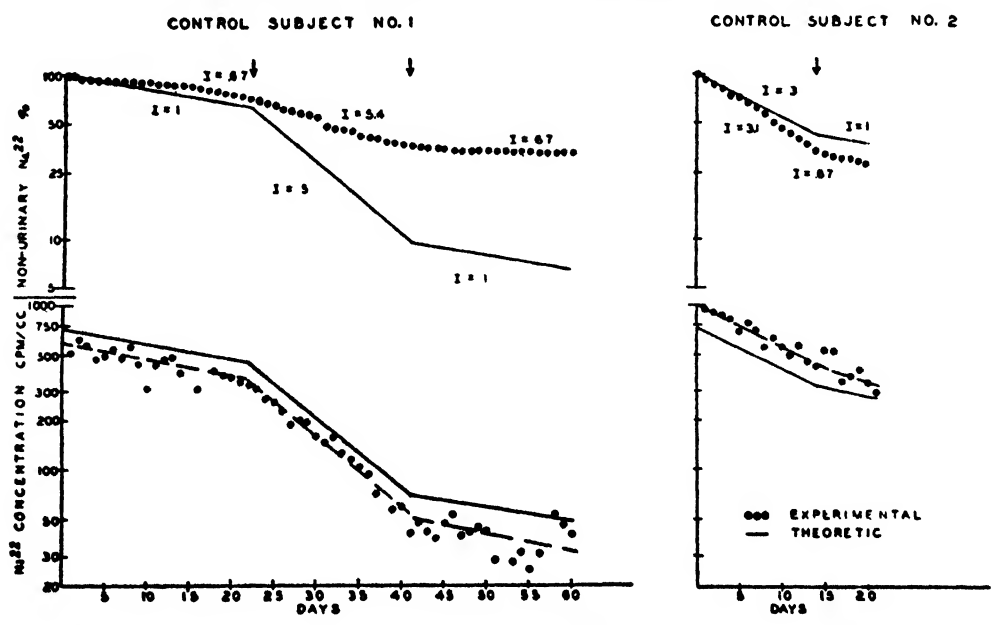

FIG. 9. Comparison of the theoretic curves of the concentration of Na^{22} and total content of Na^{22} in the body of a normal man with data obtained experimentally in 2 normal subjects. The nonurinary Na^{22} is that not excreted in the urine and is represented as percentage of total Na^{22} injected intravenously into the subject at a single dose. For the theoretic curve it represents the percentage of the total Na^{22} which is not excreted and therefore remains within the body. Values for intake of Na^{23} selected for the theoretic curves were those which approximated the dietary intake in the subjects studied. The theoretic and experimental curves agree: discrepancies between the nonurinary Na^{22} curves are due to failure to collect all excretion from the subjects studied experimentally. The experiments were designed for urinary collections alone.

General Discussion

The theoretic data presented indicate the complexities incident to an attempt to study rates of turnover from observations on the concentration within a compartment. This is particularly true in diseased states. For example, the changing size of the compartment for sodium in the subjects with generalized edema certainly makes it difficult to compare the rates of turnover in the normal subjects with those in the patients with congestive heart failure, if the serum concentration for Na^{22} is to be followed. When a patient is losing edema, $B_{1/2}$ would be smaller (the rate of elimination greater) than $C_{1/2}$ would suggest, whereas in a subject whose edema is increasing, $B_{1/2}$ would exceed that indicated by the change in serum concentration. When the state of the edema is constant or the size of the compartment is not changing, then $B_{1/2}$ could be obtained from the serum concentration, provided, of course, there were no shifts in the sodium among the various compartments. Therefore, caution should be employed whenever $C_{1/2}$ values are interpreted as evidence for $B_{1/2}$.

The great influence of the rates of intake and output of the non-tracer substance upon the elimination of the tracer substance renders necessary the consideration of diet, drugs and other measures which influence the movement of the non-tracer substance. Obviously, under most circumstances only an average $B_{1/2}$, $C_{1/2}$ or $U_{1/2}$ can be determined. In the case of sodium the custom of eating three meals will have its influence upon these parameters during the day. Such variations from absolute equilibrium and smoothness of intake may be inconsequential for most biologic purposes, but for others it can have considerable significance.

Measurements of various biologic decay rates are relatively simple for elements, but they become more difficult when molecules or more complex substances are being traced. In special circumstances, however, such as use of N^{15} to trace the life of the erythrocyte, the problem appears to be simplified by the complex structural and peculiar biologic nature of the erythrocyte. On the other hand, Fe^{59} and certainly P^{32} have metabolic characteristics which do not lend themselves as well to the tracing of the life of the human erythrocyte.

In spite of the many complex physicochemical processes and other problems involved in the metabolism of sodium, the experimental data obtained for the change in serum concentration of Na^{22} in normal man with varying intakes of sodium agree well with the theoretic calculations based on the equations presented previously (Fig. 9). Lack of agreement between $U_{1/2}$ and the theoretic $B_{1/2}$ is due to failure to measure directly all of the Na^{22} eliminated; only the Na^{22} excreted in the urine was determined. Experiments carefully designed for the quantitative measurement of the $B_{1/2}$ should be conducted.

Values of $C_{1/2}$ and $U_{1/2}$ obtained for the subjects with chronic congestive heart failure and with the nephrotic syndrome of chronic glomerulonephritis are significant biologically and must be interpreted cautiously when compared with those parameters determined in the normal subjects or when considered with respect to $B_{1/2}$ implications. The radiobiologic significance from the point of view of safety, public health precautions, and calculation of dosage is self-evident. The values also have biochemical and physiologic significance related to sodium metabolism, rate of sodium turnover, and the better understanding of states of generalized edema.

More extensive details of these studies, including the influence of drugs and certain procedures, will appear in papers to be published elsewhere.

Summary

Rates of elimination of sodium were studied with Na^{22} in normal man and in subjects with chronic congestive heart failure or with the nephrotic syndrome of chronic glomerulonephritis. Clinical phases of the diseases varied in these patients.

The nature of the experiments made it impossible to determine directly the true biologic half-life period ($B_{1/2}$) for sodium. It was necessary to introduce new terms for biologic decay periods, such as $C_{1/2}$ (the time required for the tracer to reach one-half the initial concentration in the compartment under study), $U_{1/2}$ (the time required to eliminate one-half of the tracer material by way of the urine) and $E_{1/2}$ (the time required to eliminate one-half of the tracer substance administered). The $C_{1/2}$ values were obviously less than the $U_{1/2}$ values. In the control subjects $C_{1/2}$ could not have differed appreciably from $B_{1/2}$, whereas in the diseased subjects these parameters were dissimilar.

$C_{1/2}$ for the subjects with congestive failure was about three times greater and for the subjects with the nephrotic syndrome about five times greater than that for the controls. $U_{1/2}$ was increased to a greater extent by these diseases; $C_{1/2}$ and $U_{1/2}$ varied with the state of the diseases, dietary intake of sodium, and administration of drugs. Direct comparisons of the $C_{1/2}$ values in the edematous subjects with those of the control cannot be made when the quantity of edema fluid is varying because of the influence of change in the volume of the compartment upon the concentration of the tracer.

Equations have been derived which make it possible to predict variations in concentration and total content of a tracer substance in a compartment for various rates of intake and output of the non-tracer substance and for variation in size of the tracer compartment. These theoretic considerations demonstrate the importance of considering the rate of intake of the non-tracer and variations in the volume of the tracer compartment when $C_{1/2}$ and

$B_{1/2}$ are being determined in biologic studies. Although the present investigations were chiefly concerned with sodium, the theoretic considerations are applicable to any type of tracer, organism, or compartment. The mathematical data may prove to be of value to others interested in this field of biologic research.

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Discussion

NOONAN: The data presented by Dr. Burch, showing the decreased rate of "turnover" of sodium in patients with congestive heart failure, are of great interest. Since increased "sodium space" and decreased urinary output of sodium both act to decrease sodium turnover, I should like to ask Dr. Burch if he has information concerning the relative importance of these two mechanisms involved in the slower replacement rate of body sodium in cardiac patients.

BURCH: These data do not indicate the relative importance of sodium space and renal function in the rate of turnover of sodium. As indicated in the paper, the studies, as planned, were not primarily directed at the many factors involved in sodium turnover, but merely indicate overall rates.

DESIGN AND INTERPRETATION OF CARBON ISOTOPE EXPERIMENTS IN BACTERIAL METABOLISM

S. F. CARSON

INTRODUCTION

Although carbon isotope experiments in intermediary metabolism had, as a beginning, studies on microbial systems (Wood and collaborators, 1940, 1941a, with C^{13}; Ruben and collaborators, 1939, 1940, and Carson and Ruben, 1940 with C^{11}) the recent emphasis has been largely on animal systems.

I think it is reasonably clear that a great deal of our present understanding concerning the comparative biochemical outlook in present day biochemistry has really stemmed from the clear thinking and broad point of view of the great Dutch microbiologist Kluyver, who more than twenty years ago brought forth with Donker the concept of "Die Einheit in der Biochemie" (Kluyver and Donker, 1926). The basic principles outlined here in 1926 have many times since been used to find short-cut solutions to rather complex biochemical problems. These principles of hydrogen transfer in coupled oxidation-reduction systems are now firmly impressed on us as every day and commonplace tools of biochemical thinking. Therefore, I think it is perhaps worthwhile to remember that these principles were first worked out in a relatively short time on the basis of studies concerning a number of different microbial processes.

Since a number of the people present at this conference are concerned primarily with the biochemical reactions of animal tissues, it may be worth while to consider a few of the "type experiments" which can rather easily be conducted with microbes. One might call these "model experiments," in the sense that frequently they can be conducted in a relatively uncomplicated manner.

Although it is quite clear that microorganisms must, of necessity, carry out thousands of biochemical reactions in order to build up the constituents of their enzyme systems and cell substance, the microbes have an attitude quite different from that of animals when it comes to deciding what will be their major energy yielding reactions. In a general sense one might say that animals are rather limited in the possible source of energy yielding reactions. On the other hand, it is known that the oxidation of any one of several thousand different chemical compounds, organic and inorganic, can serve as energy source for some microbe. In the now famous study of den Dooren de Jong (1926), almost every single organic chemical available in the Delft laboratories was tested and found to serve as both a carbon and energy source for one microbe or another. On the inorganic side, it is clear that such compounds as NH_3, NO_2 and H_2S can serve as energy sources for the chemoautotrophs, and even such compounds as KCN and CO fall into this class.

A second point is the following: microbes are quite independent in regard to the end-products they can produce and during fermentation give off large quantities of a relatively small number of such products.

One can often choose, therefore, not only the starting material he wants to study but the end-product as well. Thus, by a suitable choice of the microbe, one can select a system which will carry out a conversion of a given substrate to a given end-product. It is well to remember that these conversions, especially under anaerobic conditions, go in excellent yield and are largely unaccompanied by serious side reactions.

Finally, it should be brought to attention that rather nice tricks can be played on the redox level of an anaerobic process, which make it relatively simple to bring into the limelight certain enzyme processes which would otherwise go entirely unnoticed. A model experiment of this nature will be discussed a little later on.

I would like to discuss briefly a few types of reactions which were first found in microorganisms and which led then to an elucidation of general biochemical reactions of considerable importance in many types of living systems.

1. CO_2 Utilization by Heterotrophic Systems

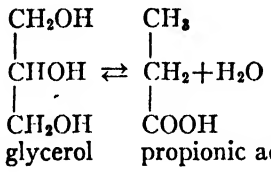
This reaction, which was discovered by H. G. Wood (Wood and Werkman, 1935, 1936), is so well known that I will not take much of your time with it but, rather, point out why it was almost necessary that this important biochemical reaction had to be first found in a microbe.

A characteristic of heterotrophs, whether they be microbes or animals, is the production of CO_2. For example, it is now known that reactions such as in Equation 1 go on simultaneously with the second one predominating, hence a *net production* of CO_2 is always observed. (Equation 1.) Therefore it is not surprising that until 1934 a clear-cut case of CO_2 utilization had not been discovered. That this was

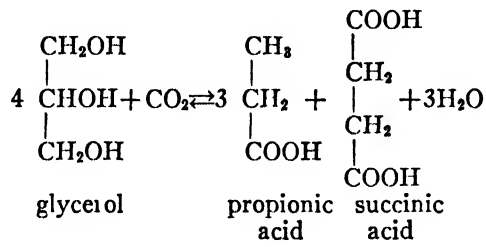
$$A + CO_2 \rightarrow B$$
$$C \rightarrow CO_2 + H_2O$$

accomplished was due only to the fact that Wood was studying the propionic acid fermentation, which

gives propionic acid in almost 100 percent yield from glycerol in a medium containing phosphate as a buffer (van Niel, 1928). (Equation 2.) When Wood used large amounts of $CaCO_3$ as a buffer (in



the presence of phosphate), a new end-product appeared, namely succinic acid. (Equation 3.) For every mole of succinate formed, a mole of CO_2



disappeared. Hard as this was to believe, the analyses were quite clear-cut and Wood had the first evidence of CO_2 utilization by a typical heterotroph. It is reasonably clear, then, that the synthesis of the 4 carbon succinate was due to a CO_2 pick-up. Subsequent isotope experiments with labelled CO_2 not only checked this result, but indicated a possible mechanism and turned up an *entirely unsuspected result*. The succinate was found to be carboxyl-labeled; but the real surprise was that the propionate was labeled, and in the carboxyl group. Some further consideration of this fermentation will be made a little later.

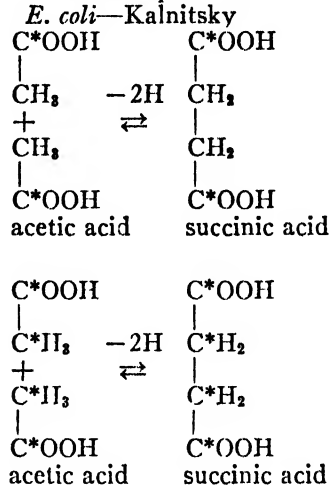
These experiments led to the finding by both the Berkeley group (Barker and Kamen, 1945; Barker, Kamen and Haas, 1945; Carson and Ruben, 1940; Ruben and Kamen, 1940) and by Wood and collaborators (1940, 1941a) that all heterotrophic systems tested utilized CO_2, whether they were microbal or animal; and we then had the fine quantitative studies by Wood and colleagues (1940, 1941a,b,c,d) on many heterotrophic systems. Subsequently it was found that CO_2 pick-up is involved in such an important system as the Krebs cycle (Wood, Werkman, Hemingway and Nier, 1941c), and eventually we have the truly magnificent studies of Ochoa and his group (Ochoa 1945, 1948; Ochoa and Weisz-Tabori, 1945) on a number of reactions in this cycle. In all fairness it should be pointed out that Ochoa apparently does not need isotopes.

2. *Thunberg-Wieland Acetate Condensation*

The condensation of acetate to succinate with concomitant dehydrogenation had been suggested by Wieland more than 25 years ago (Wieland, 1922, 1933) as a possible pathway for acetate oxidation in animal tissues. Until very recently there had never been a good indication that any biological system can carry out such a reaction.

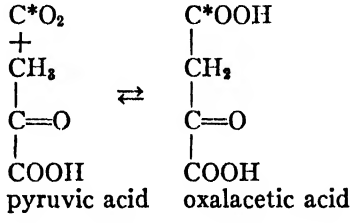
There now has been a fairly strong suggestion, with both an *Aerobacter sp.* and *E. coli*, that the following reversible reaction may take place in biological systems. (Equation 4.)

Aerobacter sp.—Slade
E. coli—Kalnitsky



3. *Oxalacetate Formation from Pyruvate and CO_2*

This exceedingly important carboxylation reaction was first suspected from the isotope work on the propionic acid fermentation, and the first real proof came from the studies of Krampitz, *et al.* (1941, 1943) on enzyme preparations from *M. lysodciktic-us*. (Equation 5.)



These studies led directly to experiments on animal tissues and, more recently, on green plants. Since this carboxylation reaction is a key point in maintaining one of the components of the Krebs cycle, it was important to have the initial unequivocal evidence obtained by Krampitz on the bacterial "model system."

4. *Oxidative Decarboxylation of Pyruvate*

The formation of acetyl phosphate as a probable intermediate on the path to acetic acid was first demonstrated by Lipmann on dried cell preparations

of *L. delbrückii* (Lipmann, 1937, 1940). The reversal of this type of reaction was demonstrated by Lipmann *et al.* (Utter, Lipmann and Werkman, 1945) using $CH_3C^{18}OOH$ and $HC^{18}OOH$ on active juices prepared from *E. coli.* (Equation 6.)

$$\underset{\text{pyruvic acid}}{CH_3\cdot C{=}O\cdot C^*OOH} \rightleftarrows \underset{\text{acetic acid}}{CH_3\cdot COOH} + \underset{\text{formic acid}}{HC^*OOH}$$

$$CH_3\cdot C^*\text{-}O\cdot COOH \rightleftarrows CH_3\cdot C^*OOH + HCOOH$$
(In presence of adenyl pyrophosphate)

It has recently become somewhat uncertain that acetyl phosphate is the actual intermediate involved and most people prefer now to speak of an "active C_2 fragment."

hand, a biological mechanism for the formation of some of the even-numbered saturated fatty acids has been worked out.

Therefore, it was felt that it would be worth while to reinvestigate this problem of the formation of propionic acid as a continuation of our initial studies made a number of years ago at Berkeley (Carson, Foster, Ruben and Barker, 1941; Carson and Ruben, 1940), and those of Wood and coworkers (1941 b,d) at Iowa. The general overall reactions of the propionic acid fermentation, showing the dependence of the direction of the reactions upon the hydrogen balance (Barker and Lipmann, 1944; Chaix-Audemard, 1940; van Niel, 1928), or the state of oxidation of the substrates, are shown in Equation 7:

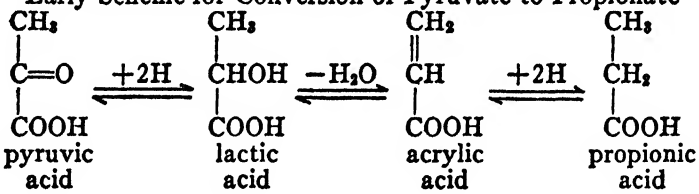
Propionic Acid Fermentation

Redox State	
(+1)	4 Glycerol + CO_2 → 3 Propionate + Succinate + 3 H_2O
(0)	$1\frac{1}{2}$ Glycerol + $1\frac{1}{2}$ pyruvate → 2 Propionate + Acetate + CO_2 + H_2O
(−1)	3 Pyruvate + H_2O → Propionate + 2 Acetate + 2 CO_2

The splendid work of Barker and coworkers on acetate metabolism and synthesis of butyric and caproic acids (Barker, 1947; Barker and collaborators, 1945) was discussed at some length by Barker

One of the older schemes proposed for the formation of propionic acid from pyruvic acid suggested lactic acid and acrylic acid as intermediates in the conversion. (Equation 8.)

Early Scheme for Conversion of Pyruvate to Propionate



at the Wisconsin Symposium last Fall, so it is hardly necessary to describe this again here.

Rather, the examples given above have been brought up in order to emphasize the point that one or another microbial system can usually be found which will serve as a model system for the study of certain fundamental biochemical reactions. These results have almost invariably led to similar or equivalent findings in the more complicated animal systems.

I would like now to discuss some research on the propionic acid fermentation which will perhaps illustrate some principles of possibilities of design and interpretation. In this work we have had the collaboration of Martin Kuna and D. S. Anthony, and the assistance of Misses Long and Bachmann. The studies were conducted on the propionic acid fermentation with *Propionibacterium pentosaceum* using C^{14} as a tracer.

It has been mentioned that the mechanism of formation of propionic acid in biological systems has always been a difficult problem. On the other

This scheme was discarded when it was not possible to show conversion of acrylate to pyruvate with dried preparations of the propionic acid bacteria and when it was clearly shown by Barker and Lipmann that NaF inhibited the conversion of lactate to propionate but did not inhibit the conversion of pyruvate to propionate (originally observed by Chaix-Audemard, 1940).

Earlier experiments by Wood, Stone and Werkman (Stone and Werkman, 1936; Wood, Stone and Werkman, 1937) have indicated that acetate can be metabolized during this fermentation. In one set of experiments acetate reduced methylene blue, and in the analyses of some fermentations there was a reasonable indication that some of the initial acetate which was formed disappeared during later stages. The isotope experiments described below give a rather definite indication that acetate is metabolized at a somewhat considerable rate. Previous tracer experiments by Wood and coworkers (1941 b,d) and the Berkeley group (Carson, Foster, Ruben and Barker, 1941; Carson and Ruben, 1940) have indi-

cated that propionate is formed by the decarboxylation of C_4 compounds which arise by the process of CO_2 fixation or by condensation of intermediate products. Therefore it seemed desirable to set up an experiment which would show the probable equilibrium relationships between substrate-acetate-propionate and to test whether or not simple conversion of pyruvate to propionate and acetate occurs reversibly with the C_3 skeleton remaining intact between pyruvate and propionate. Using substrates at three stages of oxidation, the shift in equilibrium was traced with acetate* (* indicates compound labeled with C^{14}) and CO_2* as the amount of available hydrogen varied. Thus, reactions which might appear obvious with one state of available hydrogen, but not with another, may be made clearly obvious by the shift in equilibrium.

It is realized by all of you, I think, that the complete separation of all the end-products of a biological reaction and the subsequent degradation of each compound into individual carbon fragments is a very necessary prelude to an interpretation of the most probable path of the reaction. Hence, some preliminary tracer experiments should be made in order that one can be more certain that such difficult and time-consuming experiments will not be largely wasted effort.

A basic principle of fermentation chemistry which has seldom been used, but which lends itself readily to studies of anaerobic processes, is the use of substrates of varying redox value. It is quite apparent, from the large qualitative and quantitative differences in end-products from the three different substrates shown before, that some possible enzyme reactions are utterly and completely hidden under one set of redox conditions but are able to show their effect under other conditions.

In the actual experiment, substrates of several different states of oxidation (or available H) were used: glycerol, a highly reduced substrate; pyruvate, a highly oxidized substrate; and a 1:1 mixture of glycerol and pyruvate. Pyruvate is very probably an intermediate in the conversion of glycerol to propionate and acetate. Two fermentations were carried out with each of the three substrates; one with high specific activity carboxyl labeled acetic acid* as a tracer and one with CO_2* in the form of $NaHCO_3$* as a tracer (Table 1).

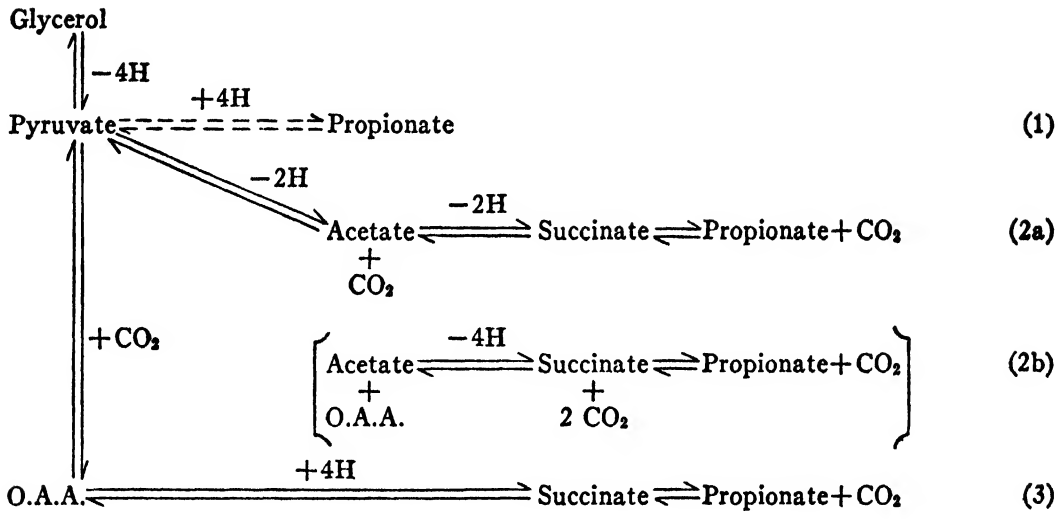
TABLE 1

Substrate	Glycerol		Glycerol+Pyruvate		Pyruvate	
Reduction Value	14	(+1)	12	(0)	10	(−1)
Tracer Added	H·Ac*	CO_2*	H·Ac*	CO_2*	H·Ac*	CO_2*
% initial C* found as CO_2*	2	66	6	73	33	58
Ratio Specific Act. $\frac{c/s}{mM}$ of $\frac{\text{Propionic}}{\text{Acetic}}$	$\frac{1}{100}$	$\frac{1}{3}$	$\frac{1}{33}$	$\frac{1}{1.7}$	$\frac{1}{4}$	$\frac{1}{0.5}$

With glycerol, a reduced substance, as substrate the ratio of the specific activity of propionate* formed to acetate* remaining is 1:100; with a mixture of glycerol and pyruvate the proportion was 1:33; while with pyruvate as substrate the ratio was 1:4. Thus the ratio of conversion of acetate* to propionate* was 25 times as great in the presence of an oxidized substrate.

With glycerol as a substrate and CO_2* as a tracer, the ratio of the specific activity of propionate* produced to that of acetate* produced was 1:3. With a mixture of glycerol and pyruvate the ratio was 1:1.7, and with pyruvate as a substrate the ratio was 1:0.5. Thus six times as much CO_2* is converted to propionate* with an oxidized substrate. The amount of the initial C^{14} added as acetate* which was converted to CO_2* was 2 percent with glycerol as substrate, 6 percent with a mixture of glycerol and pyruvate, and 33 percent with pyruvate. *This is an important indication that acetate* is metabolized through a condensation reaction which subsequently yields CO_2**. Such a reaction is not apparent with glycerol as the substrate, but with the use of an oxidized substrate (pyruvate) it obviously assumes great importance in the equilibrium scheme.

On the basis of pathways suggested by earlier as well as the present tracer experiments, we have proposed a working scheme around which one can design a more complete tracer experiment (Equation 9).



IIA_c*→Propionate*	Highest conversion with pyruvate
HA_c*→CO_2*	Highest conversion with pyruvate
CO_2*→Propionate*	Highest conversion with pyruvate
CO_2*→Acetate*	Highest conversion with glycerol

If reaction (1) ocurs: it would yield propionate* from acetate*, and propionate* from CO_2* only by reversal of the oxidative decarboxylation of pyruvate, would therefore *require* hydrogen, and would be expected to occur to the greatest extent with glycerol as the substrate.

However, the largest production of propionate* from acetate* occurred with pyruvate as the substrate with simultaneous production of CO_2*. In addition, the production of propionate* from CO_2* was favored by pyruvate. Finally, the pyruvate remaining in these fermentations (⅓ of the initial amount) did not contain any measurable amount of labeled C*.

If reaction (3) goes, it would yield propionate* from CO_2*, and would be favored by glycerol as a substrate. The production of propionate from CO_2* was favored by pyruvate which is not what one would expect.

However, if reactions (2) and (3) were occurring simultaneously this would be possible. Reaction (2a) or (2b) would yield propionate* and CO_2* from acetate* and would be favored by pyruvate. Actually the largest production of propionate* and CO_2* from acetate* occurred in the presence of pyruvate, indicating that reactions (2) were possible. Reactions (2) would proceed with the evolution of pairs of hydrogen atoms which would allow reaction (3) to proceed. This would account for the greater production of propionate* from CO_2* in the presence of pyruvate, since the hydrogen necessary to allow reaction (3) to proceed can come from reaction (2).

This may seem like a very round-about method of turning up the possible intermediates between pyruvate and propionate; however, it now begins to become somewhat clear why the experiments of Barker and Lipmann were unsuccessful in demonstrating lactate or acrylate as intermediates. One of the redox conditions under which the present experiments were conducted demonstrated that acetate can be converted to propionate and CO_2; therefore, one is almost forced to look for an acetate condensation. In addition, there is now excellent comparative biochemical evidence for a reaction involving the decarboxylation of succinate to propionate and CO_2.

Summary

One might say, first, that microorganisms can be, and have been, used as model systems to test certain fundamental types of biochemical reactions. Secondly, that advantage can be taken under anaerobic conditions to change the state of the redox level at will in order to make evident reactions which otherwise might be completely hidden, and that this can be accomplished without recourse to addition of foreign substances such as redox dyes. This advantage may then be used to design complex tracer experiments by looking over the possible reactions and then choosing the most ideal conditions under which to carry out the difficult and time-consuming radiochemical separations and degradations.

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AMINO ACID METABOLISM IN *TORULOPSIS UTILIS*

GÖSTA EHRENSVÄRD

With G. Högström, L. Reio, E. Sperber and E. Saluste[1]

During the last years Torulopsis yeast has become a very suitable object for the study of amino acid metabolism in connection with protein synthesis. The work of Fink and collaborators (1936 a and b) made it possible to cultivate *Torulopsis utilis* on a laboratory scale, producing 100 to 200 g. in an eight hour experiment. Later Sperber (1945), in an extensive investigation, established a reproducible technique for preparing Torulopsis yeast with low nitrogen content (4-5%) compared with ordinary Torulopsis with a nitrogen level of eight to nine percent. Most important are his findings that this low-nitrogen yeast is able to attack a wide range of nitrogenous substances, utilizing the nitrogen to raise the low nitrogen level to the higher stage, *even if no exogenous carbohydrates are present as substrate.*

The technique of preparing low-nitrogen Torulopsis consists in cultivating high-nitrogen yeast on a nitrogen-free medium containing 50 g. sucrose, 3 g. KH_2PO_4, 1 g. magnesium sulphate and 0.5 g. calcium chloride per liter of tap water. In about seven hours the high-nitrogen yeast reduces its nitrogen content to five to six percent and is then ready to make up for the nitrogen deficit by metabolizing an amazing number of substances, such as ethylamine, benzylamine, guanidine, nicotinamide and most amino acids. In some of these cases the substances, after being subject to deamination or transamination processes, are utilized by the yeast as carbon sources for growth.

The formation of low-nitrogen Torulopsis has later been investigated by Roine (1947), whose work has mainly centered on the nature of the primary nitrogenous products formed in the yeast cells during the first phases of ammonia uptake by low-nitrogen Torulopsis. Together the works of Sperber and Roine, covering the literature on Torulopsis metabolism up to 1946, give a fairly clear outline of the first stages of the nitrogen utilization by the yeast, a summary of which may be stated as follows:

1. During the cultivation of high-nitrogen yeast on a carbohydrate diet in the absence of exogenous nitrogen sources, the high-nitrogen level remains constant until three hours after the start of the experiment, whereafter it falls to the six percent level over two hours. A further slow descent to a four to five percent N-level is observed in the next three hours.

2. The yeast quantity slowly drops during the first three hours of cultivation, whereafter it rises, reaching a steady level at the six-hour point. After all endogenous ammonia sources have been exhausted, budding decreases rapidly, becoming insignificant at 4.5 hours.

3. In *aerated* cultures of low nitrogen yeast, fed with ammonium sulphate and sucrose, a distinct rise of the nitrogen level is observed after one hour. By this time budding has begun, and is usually already observable after half an hour. Nearly all of the cells are dividing after three hours, by which time the total nitrogen content of the yeast has increased half-way towards the high-nitrogen level.

On the other hand, in experiments of a similar kind but *without* any exogenous carbohydrate source no budding is observable until four hours after the addition of a utilizable nitrogenous substance. In any case, according to Roine (*loc. cit.*), the nitrogen uptake nearly ceases at 1.5 hours, at which time most of the endogenous carbohydrates have been utilized.

4. In non-aerated cultures only a very slight nitrogen uptake is observable (Roine, *loc. cit.*).

5. Part of the nitrogen taken up from the medium was found in the trichloracetic acid-soluble fraction of the yeast, this fraction containing amino dicarboxylic acids and their amides, together with alanine. Glutamine seems to be the main amide component.

6. Transamination systems observable in Torulopsis homogenates are:

Glutamic acid ..... Pyruvic acid

Aspartic acid, Alanine, Valine, Leucine, Isoleucine ..... α-ketoglutaric acid

The facts summarized above form the basis for the present investigation, which covers some special features of the utilization by Torulopsis of alanine, glycine, and carbon dioxide.

Preliminary Experiments

With the knowledge that DL-alanine and glycine can be used as nitrogen sources for low-nitrogen Torulopsis (Sperber, 1945) we wished to discover to what extent this nitrogen uptake could be accounted for as (1) uptake of ammonia N, resulting from deamination; (2) formation of amino acids by

[1] Our thanks are due to B. Johansson, B. Liljequist and H. v. Ubisch for carrying out the mass-spectrometer measurements. We also wish to express our thanks to the Swedish Scientific and Medical Councils for financial support.

TABLE 1. AERATION OF LOW-NITROGEN TORULOPSIS YEAST WITH ADDITION OF LABELED CO_2

81 g. wet yeast = 23 g. dry weight. 5.17% N.
982 ml. CO_2 (2.43% C^{13} excess) passed through suspension during 150 min. 400 mg. ammonium sulphate added at start. Temperature in this and the following experiments 30°.

	mg. total carbon	Atom % C^{13} excess	mg. C^{13} excess
Total CO_2 in air current after passage through yeast suspension	1315	0.72	9.50±0.03
Yeast hydrolysate			
Water soluble fraction (mainly amino acids)	6870	0.025	1.72±0.10
Water insoluble fraction (mainly humin)	2480	0.027	0.67±0.07
Total C^{13} excess recovered			11.89±0.20
Total C^{13} added as CO_2	525	2.43	12.75±0.10

transamination of keto acids; (3) incorporation of the amino acid of the substrate into the protein framework, directly or indirectly.

The third question is closely related to the problem of whether the carbon skeleton of alanine and glycine—after deamination—could be utilized as such, or in part, as components of the protein synthesis during the process of nitrogen uptake. To that end some preliminary experiments were made with DL-alanine, DL-glutamic acid, and glycine, all labeled in the carboxyl group with an excess C^{13}, using each of these amino acids as sole substrate for low-nitrogen Torulopsis yeast. Some of these experiments have been described (Ehrensvärd and coworkers, 1947), and show that a considerable amount of the labeled carboxyl groups appears in the respiratory CO_2. Some of the C^{13} excess originating from the metabolized substrates was however found to be fixed by the yeast, mainly as carboxyl groups of the amino acid fractions, isolated after hydrolysis. The rather even distribution of C^{13} excess among the amino acid fractions emphasized the necessity of investigating the potentialities of low-nitrogen Torulopsis yeast concerning over-all CO_2-fixation, as well as the need of working with D and L forms of the amino acids separately, when using them as substrates.

CO_2-FIXATION

In order to investigate the CO_2-fixation of Torulopsis, an experiment was carried out in which a constant amount of C^{13}-labeled CO_2, liberated from $BaC^{13}O_3$ by slow and constant addition of 1 N HCl, was added to the air current to be passed through the yeast suspension. The air current was previously freed from CO_2 by passing through strong KOH solution. In addition to CO_2, the yeast was given a certain amount of ammonium sulphate to stimulate growth, the yeast being low-nitrogen Torulopsis, prepared three hours previously. After passage through the yeast suspension the gases were passed into baryta solution, trapping all CO_2 present as $BaCO_3$, from which CO_2 was later liberated and analyzed for its C^{13} content.

The mass-spectrometer values of this and the following experiments have an average error, corresponding to a maximal spread of ±0.003 in the range of 0.020 to 0.050 percent C^{13} excess. For figures higher than 0.050 percent the average spread will be correspondingly higher. Values between 0.000 and 0.010 percent are recorded as <0.01 percent.

The C^{13} turnover of the C^*O_2-experiment, carried out over a period of two and one half hours, is shown in Table 1.

The experiment shows that during the period of nitrogen uptake the yeast was able to retain about 20 percent of the C^*O_2 passed through the suspension. The labeled carbon atoms of the CO_2 appear for the most part in the water-soluble fraction of the yeast hydrolysate, and there mainly in the carboxyls of the amino acids present. The distribu-

TABLE 2. CONDITIONS EMPLOYED FOR 5 EXPERIMENTS WITH AMINO ACID UPTAKE BY LOW-NITROGEN TORULOPSIS YEAST

Time of experiment, 3 hours

Substrate	Alanine-C* (1) D(−)	Alanine-C* (1) L(+)	Alanine-C* (2) D(−)	Alanine-C* (2) L(+)	Glycine-C* (1)
% C^{13}—excess in labeled atom	1.83	1.83	1.26	1.26	1.68
g. yeast					
wet weight	46	46	60	60	40
dry weight	12.5	12.5	16.1	16.1	10.5
g. amino acid added as substrate	2.3	2.4	3.0	3.0	2.0
mg. C^{13} excess					
1. In the labeled amino acid added	5.63	5.92	5.09	5.09	5.40
2. In respiratory CO_2	3.10	4.67	0.28	1.05	1.47
3. Fixed in yeast, estimated by combustion	2.24	1.35	4.85	3.45	1.20

tion of retained C^{13} excess in the individual amino acid fractions is shown in connection with the following experiments, where carboxyl-labeled glycine and D(−) and L(+) alanine were used as sole carbon substrates.

The Uptake of Carboxyl-Labeled Glycine, D(−) and L(+) Alanine

Three sets of experiments were made, one with glycine, the others with D(−) and L(+) alanine, carried out under identical conditions. The C^{13} excess of the carboxyl groups, the properties of the yeast employed, and the amount of yeast and amino acids is shown in Table 2. The amino acids were added at start of the experiment, and from time to time the amount of respiratory CO_2 was collected as $BaCO_3$, which was analyzed as to its C^{13} content. From these values, the weights of $BaCO_3$, and the actual C^{13} excess of the substrate, it was possible to calculate *the amount of utilized carboxyl carbon as a percentage of the total carbon of the respiratory CO_2 during the same interval.* The variation of this percentage at different stages of the experiment is shown in Fig. 1; in the curves marked C*(1). (The C*(2)-curves belong to a set of experiments to be described later.)

From the diagram it can be seen that the metabolic uptake of glycine and the alanines differ in certain respects. Whereas the glycine carboxyl appears steadily at an increasing rate in the respiratory CO_2 and then falls off after three to four hours, the alanines give off their carboxyl carbon more readily during the first 45 minutes, whereafter the percentage of carboxyl output remains constant until about two hours after the start of the experiment. Then an increased liberation of carboxyl C as CO_2 takes place, reaching a maximum at three hours, after which the C^{13} level of the respiratory CO_2 rapidly drops.

This behavior of glycine and the alanines is in accordance with the findings of Sperber (1945, p. 65) who states that glycine is metabolized at a much slower rate than DL-alanine, with little or no inhibition of endogenous respiration, and that DL-alanine after a rapid preliminary uptake exhibits a period of induction of about two hours before entering the final stage of increased metabolic breakdown. The question is, however, far from simple in that, after splitting off nitrogen from the substrate, part of the carbon moiety is utilized for synthetic purposes, affecting in the same period of time the rate of endogenous respiration. From our experiments the latter is definitely different in the case of the uptake of D(−) and L(+) alanine.

In order to investigate the distribution of C^{13} excess in the carboxyl groups of the amino acid residues of the yeast, the latter, after being in contact with and having metabolized part of the labeled substrate (the substrate being $CO_2 + NH_3$, or glycine, or D(−) and L(+) alanine), was separated, washed and hydrolized with 20 percent HCl. Working up of the hydrolysate was accomplished by removing HCl, electrodialyzing to separate the basic,

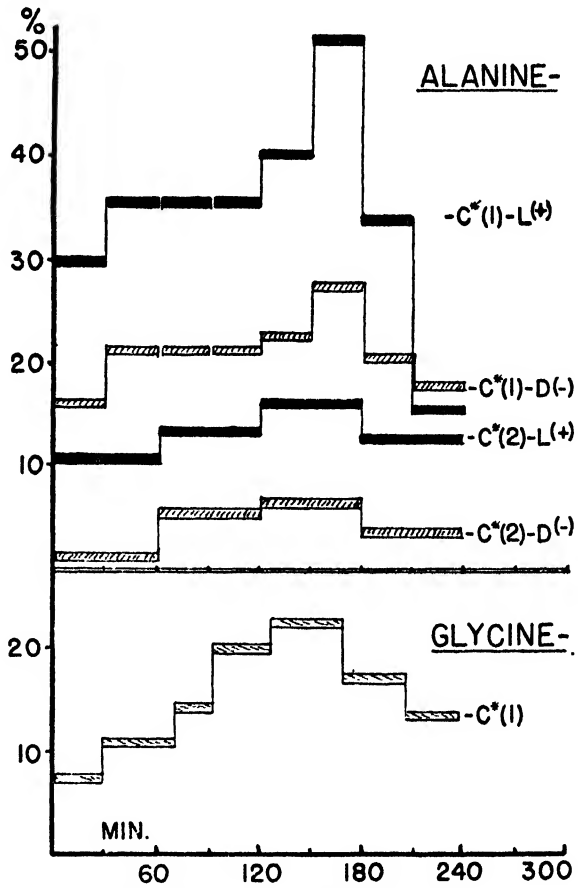

FIG. 1. Five sets of experiments, showing the percentage of the C(1) and C(2) atoms of D(−) and L(+) alanine, and the C(1) atom of glycine, in the total carbon content of respiratory CO_2 measured at intervals. For conditions of the experiments see Table 2.

acidic and neutral fractions (Sperber, 1946) and isolating the amino acids as:

Arginine-monoflavianate
Histidine-nitranilate
Lysine-picrate
Glutamic acid hydrochloride
Aspartic acid-copper salt
Glycine nitranilate
Alanine-azobenzene-p-sulphonate
Serine-p-hydroxy-azobenzene-p-sulphonate
Proline-copper salt
Hydroxy-proline-copper salt
Valine/isoleucine-copper salt
Leucine/phenylalanine/methionine-copper salt
Tyrosine
Cysteine-copper complex.

Of the substances above, all are in pure state as tested with the paper chromatogram method except the valine- and leucine-containing fractions which are isolated in a crude state. The isolation of the components of these fractions, together with details of the total isolation technique, are to be published in the near future.

As to the main procedures of the amino acid isolation the working up of the basic fraction was done by a modification of the method of Kossel, worked out by Bloch and Bolling (1945). The same authors' method for separation of aspartic acid and glutamic acid was used, whereas the neutral fraction was subjected to separation by means of a modification of the copper salt method outlined by Town (1936) and Brazier (1930). Some of the amino acids of the latter fraction were isolated according to the methods of Town (1928), and Stein and coworkers (1942). The carboxyls of the different amino acid fractions were liberated by ninhydrin treatment, using the method of Van Slyke (1943), whereby aspartic acid yielded *both* of its carboxyls as CO_2. The glycine and the serine salts were freed from the acid component before ninhydrin treatment.

The result of the fractionation of the hydrolysates from the CO_2, glycine and alanine experiments is shown in Table 3. Surveying the figures indicating C^{13} excess determined in α-carboxyl of isolated amino acid fractions, some features of interest might be recorded:

1. The C^{13} excess coming from labeled CO_2 is preferentially trapped in the acidic fraction, especially in one (or both) carboxyls of aspartic acid, being most probably the result of carboxylation of pyruvic acid to oxalacetic acid, followed by transamination. This observation indicates that, in all cases where labeled CO_2 results from the metabolism of a C-labeled substance, carboxylation is likely to contribute to some extent to the accumulation of isotope-excess in amino acid carboxyls, especially in the acidic fraction.

2. Concerning the experiments with D(−) and L(+) alanine it is not surprising that the overall distribution of C^{13} excess in the various fractions shows a definite difference, considering the possibilities of the two isomers having different metabolic patterns. It is, however, interesting to note that, in spite of its higher decarboxylation rate (see Fig. 1), L(+) alanine contributes about double the amount of C^{13} excess in the ninhydrin-liberated CO_2 from the total hydrolysate, as compared with its D(−) isomer, and that most of its C^{13} excess appears in the glutamic acid α-carboxyl. Some of the latter might

TABLE 3. ATOM PERCENT OF C^{13} EXCESS IN TOTAL C AND CARBOXYL GROUPS (C(1)) OF AMINO ACIDS ISOLATED FROM HYDROLYSATES OF TORULOPSIS YEAST

Four different experiments: Glycine-C*(1), Alanine-C*(1), Alanine-C*(2) and C^*O_2 ($+NH_3$) used as single carbon substrate for growing low-N Torulopsis yeast. Bold-face figures for parallel D(−) and L(+) series indicate the higher turnover rate of the series. In the case of aspartic acid the figures indicate the sum of the values for *both* carboxyls. — indicates: no data available.

Substrates in medium	Glycine-C*(1)	Alanine-C*(1)		Alanine-C*(2)				$C^*O_2^-(+NH_3)$
Percent C^{13} excess in labeled atom of the substrate	1.68	1.87		1.26				2.43
Series		D(−)	L(+)	D(−)	L(+)	D(−)	L(+)	
Carbon atoms isolated from amino acids of the hydrolysates	C(1)	C(1)	C(1)	Total carbon		C(1)	C(1)	C(1)
Arginine	.01	.11	**.14**	.07	**.11**	.01	**.01**	.04
Histidine	.01	.01	**.01**	.03	**.05**	.01	**.01**	.01
Lysine	.03	.01	**.015**	—	—	.01	**.01**	.01
Aspartic acid	.03	.12	**.15**	.06	**.09**	.01	**.06**	.08
Glutamic acid	.06	.14	**.23**	.03	**.16**	.02	**.12**	.04
Tyrosine	.06	.03	**.06**	.025	**.06**	.02	.005	.01
Glycine	.74	.02	**.06**	.09	**.13**			.02
Alanine	.01	**.31**	.06	**.25**	.16	(Total neutral		.025
Serine	.18	**.33**	.09	—	—	fraction)		.03
Proline	.12	.11	.06	—	—	—	—	.01
Valine-isoleucine	.20	**.18**	.12	**.11**	.08	—	—	.02
Leucine-phenyl alanine methionine	.06	.09	.04	**.14**	.09	—	—	.01
	Percent C^{13} excess in isolated carbon atoms of amino acids							

arise from secondary fixation of the liberated respiratory CO_2, which contains a considerable amount of C^{13}; the whole amount of the labeled glutamic acid carboxyl is, however, too large to be accounted for as the result of CO_2-fixation, and might be correlated with the processes connected with pyruvic acid metabolism.

The high turnover rate of arginine carboxyl is somewhat surprising in view of the low C^{13} content of the other basic amino acids.

3. As to the glycine experiments, the appearance of C^{13} excess in the isolated glycine, glutamic acid and aspartic acid reflects a similar course of glycine uptake as in the experiments of Greenberg and Winnick (1948) on rats and in those of Shemin on the human (in press). Its appearance in our experiments in isolated serine, proline and the crude valine-isoleucine fraction may be a special feature of Torulopsis metabolism. Care was taken in the glycine experiments to purify the fractions isolated to complete freedom from contaminating glycine, three times recrystallizing the serine-p-hydroxy-azobenzene-p'-sulphonate, and isolating the proline over rhodanilate, then over the Cd-complex and the copper salt. No ninhydrin color could be developed from the final proline preparation, indicating absence of all other amino acids. The preferential appearance of the labeled glycine of the substrate in the isolated glycine of the hydrolysate indicates, if fully confirmed by similar results from a number of experiments with other biological systems, that a specially rapid exchange exists between the glycine of the substrate and protein-bound glycine. No attempt to explain this behavior can at present be made.

The Uptake of C(2)-Labeled D(−) and L(+) Alanine

In view of the difficulties of obtaining any clear-cut results from the experiments with carboxyl-labeled D(−) and L(+) alanine, these amino acids were synthesized with an excess of C^{13} in the (2)-position. The method employed for this purpose was developed by Baddiley, Ehrensvärd and Nilsson (in press), yielding C(2)-labeled D L-benzoyl-alanine, which was subjected to resolution with the aid of strychnine and brucine in the manner described by Pacsu and Mullen (1940).

The Torulopsis experiment was carried out in the same way as the previous experiments with carboxyl-labeled alanines. The conditions of the experiment, the amount of yeast and amino acids are shown in Table 2. During the experiment samples of respiratory CO_2 were taken at intervals and analyzed for their C^{13} excess.

The C^{13} excess of the respiratory CO_2 is in this case certainly a measure of the extent to which the alanines have been totally metabolized. In the diagram, Fig. 1, the percentage of C^*O_2 coming from labeled carboxyl of the substrate, relative to total amount of respiratory CO_2, has been calculated, knowing the isotope excess of the labeled group of the substrate and that of the CO_2, measuring the weight of the latter (as $BaCO_3$) during the course of the experiment. Judging from the diagram it appears that the C^*O_2 output, emanating from *both* the C*(1) and the C*(2) atoms from the L(+) series are considerably higher than the corresponding amount from the D(−) series, which indicates that L(+) alanine definitely has been subject to a higher rate of total oxidation than has D(−) alanine. The rest of the C^{13} excess taken up from the medium but not appearing in respiratory CO_2 ought to be found incorporated in the yeast substance, appearing after hydrolysis in some of the amino acid fractions, whereby the C*(2)-D(−) series ought to contain more total C^{13} excess than the C*(2)-L(+) series. The figures of Table 4 show that this is the case.

In both series the C^{13} excess is mainly localized in the *non-carboxylic* parts of the amino acid fractions isolated. The C^{13} content of the carboxyls is considerably higher in the L(+) series, compared with the D(−) series, being most likely the result of secondary carboxylation with labeled respiratory CO_2. The preferential labeling of the carboxyls of the acidic fraction is in accordance with this view.

Concerning the differences of isotope excess of the individual amino acids isolated (estimated by total combustion), no general qualitative difference appears between the D(−) and L(+) series. On the other hand, the quantitative difference between the pronounced accumulation of C^{13} excess in the neutral fraction of the D(−) series, as compared with the even isotope distribution of the L(+) series, gives some hints as to the possibilities of two different pathways of metabolic breakdown of the D(−) and L(+) isomers.

Since the *average rate of nitrogen uptake* is almost the same in the two series, one arrives at the conclusion that the main features of the pattern of C^{13} incorporation may take place through the same reaction products, appearing at an early stage in the deamination process. Even if this process would not necessarily be the same for D(−) and L(+) alanine, but could occur by different mechanisms, each of these might yield two reaction products, both of the same kind in both series. It is likely that these two reaction products might be identified as pyruvic acid on the one hand and acetic acid (or possibly acetaldehyde) on the other, appearing in the metabolism of D(−) alanine at a *different ratio,* as compared with that of the L(+) isomer. Such a quantitative difference of the primary stages of D(−) and L(+) alanine metabolism might in turn be reflected in the average pattern of C^{13} turnover, as shown in the C*(1) and the C*(2) experiments.

Discussion

In reviewing our experiment with Torulopsis some

warning must be given as to generalization of the results obtained when compared with similar experiments on other biological systems. The remarkable adaptability of *Torulopsis utilis* to different substrates stands in many respects in direct contrast to the marked dependence on a specific diet of the cells of animal tissues. A generalization which could be made about the experiments recorded here is that they clearly point to some precautions that must be taken into consideration in future work of this kind.

First, the common feature of the experiments, namely, the more or less predominant presence of split products from the substrate amino acid in the amino acid of the same type isolated from the yeast proteins is worth special attention. Until proved that the observed high isotope excess is not solely the net result of a certain amount of non-metabolized labeled substrate, accumulating in the intra- or intercellular water phase, it is not possible to state with certainty that there has been a preferential incorporation of the amino acid residue in question. In the case of the Torulopsis experiment with glycine the following calculation shows to what extent non-metabolized substrate will interfere with the figures obtained for the C^{13} excess of glycine and the other amino acids isolated.

In the glycine C*(1) experiment (Table 3) about 40 g. of yeast is separated after the end of the experiment, from a substrate solution containing about 870 mg. non-metabolized glycine in 900 ml. of water. The isotope excess of the carboxyl is 1.68 percent. The amount of yeast separated corresponds to a total water content of at most 30 ml. Making the pessimistic assumption that the concentration of glycine in this inter- and intracellular water is the same as that of the medium, one arrives at the figure of 29 mg. of free glycine separated together with the yeast. Now however the yeast is thoroughly stirred up with water, letting the suspension settle and then subjecting it to hard centrifugation. The amount of glycine remaining in the intercellular space after this treatment will be insignificant. As to the amount of glycine still dissolved in the intracellular water, there would remain about 20 mg., some of which however will dialyze out in the surrounding medium during the washing and the centrifugation, some being metabolized (slowly, on account of the absence of aeration). In view of the fact that 1130 mg. of glycine have been metabolized during three hours, it is reasonable to assume that most of the remaining 20 mg. will be metabolized during the one to two hours of washing procedures. Now 350 mg. of glycine nitranilate is isolated from the yeast hydrolysate, corresponding to 140 mg. of glycine. Assuming five to ten mg. of substrate glycine, the latter would contribute 0.06 to 0.12 percent of isotope excess in the carboxyl of the isolated glycine. The actual figure found is 0.74 percent, which makes it possible to state with some confidence that the carboxyl-labeling of the glycine isolated is mainly due to metabolic transitions of the glycine substrate. In any event, the possibility of intracellular accumulation of labeled substrate as outlined above is a factor that should be kept in mind for other cases of this kind.

Another question is worthy of consideration. From the fact that labeled CO_2 is readily fixed in the protein, appearing after hydrolysis as α-carboxyl, mainly in aspartic acid, it is evident that any substrate yielding labeled CO_2 on metabolic break-down will cause a secondary over-all labeling of many amino acid carboxyl groups. This implies some undesirable complications in the study of the metabolic fate of carboxyl-labeled amino acids. C(2)-labeling, although more elaborate to carry out, is preferable, especially in investigations on the metabolic transitions of one amino acid into others. Double labeling of C(2) and C(3) with different carbon isotopes, for example, would naturally be the most desirable procedure for such investigations.

It may finally be pointed out that, not knowing the rate of endogenous respiration in each special case, it is not possible to carry out any quantitative measurements of the rate at which different atoms of the substrate are given off as CO_2 or temporarily used for synthetical purposes. Many observations (*cf.* Borei, 1942) point to the fact that endogenous respiration could never be ascribed to any "basal" value, but is changing according to experimental conditions and the special configuration of the substrate added.

In order to separate the effect of metabolic breakdown of the endogenous substrate from that of the exogenous taken up from the medium, each experiment with partially labeled substrates should be run parallel with a control containing the same substrate uniformly labeled in all carbon atoms. In such an experiment the net amount of CO_2 from respiration and the isotope excess would give a definitive answer as to the rate of respiration of endogenous material and that of the substrate during the whole course of the experiment.

In the case of D(−) and L(+) alanine an experiment of this kind has to be done, the outline of which embraces labeling of the alanines in C(2) with C^{13}, and in C(3) with C^{14}, using a N^{15}-labeled alanine of the same optical configuration, uniformly labeled in all three carbon atoms, as a control of endogenous respiration and nitrogen turnover. Such an experiment is likely to give a more complete view of the different metabolic pathways of the alanines in Torulopsis; a special case, however, of one of the many biological objects to be investigated. The necessity for carrying out series of similar experiments with many amino acids over the wide range of biological systems, simultaneously correlating the amino acid metabolism with that of carbohydrates, purines and pyrimidines indicates the somewhat arduous aspect of what has to be done in order to reveal some of the secrets of protein synthesis.

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STUDIES ON CAPILLARY PERMEABILITY WITH TRACER SUBSTANCES

LOUIS B. FLEXNER, DEAN B. COWIE AND GILBERT J. VOSBURGH

The unique advantages of the tracer technique for measuring capillary permeability have been clearly stated by Hahn and Hevesy (1941). Let us suppose that we wish to measure the rate of escape of the sodium ion from the blood plasma across the capillary wall into the extravascular fluid. We might propose to do this by injecting sodium chloride intravenously and by measuring the subsequent decline in concentration of sodium in the plasma. This approach would carry with it, however, two uncertainties. Unless the normal level of sodium in the plasma were considerably exceeded, quantitative analysis for excess sodium would present difficulties and excess sodium chloride in the plasma would likely be followed by a shift of water from the extravascular fluid into the plasma. Decrease in sodium concentration in the plasma would consequently be due to the additive effect of two rates, the rate of movement of water into the plasma, and the rate of escape of sodium from the plasma. Even though there were no shift of water, it would not be certain that a measure of the rate at which sodium ions cross the capillary wall would be obtained because, under the conditions of the experiment, sodium and chloride ions must move together and the resistance of the capillary wall to the two ions may be different. As Hahn and Hevesy further point out, use of the radioactive isotope of sodium administered in tracer amounts as radiosodium chloride obviates these difficulties. The total number of sodium ions (Na^{23} and Na^{24}) in the plasma remains practically constant throughout the experiment in these circumstances and measurement will be made of the rate of exchange of the radiosodium ions of the plasma with the untagged sodium ions of the extravascular fluid.

To what particular problems of capillary physiology might this approach contribute? There are four which seem of much interest to us. The first is to determine the rate at which the constituents of the plasma are exchanged with their extravascular counterparts. What proportion of the plasma sodium, for example, is exchanged per unit of time with extravascular sodium? The second is to test the hypothesis proposed, we believe, by Chambers and Zweifach (1940, 1947), accepted apparently by Danielli and Stock (1944) and questioned by Landis (1946), that water and dissolved gases pass through the whole of the capillary wall, that is through the endothelial cells as well as the intercellular cement, whereas the important avenue of escape for electrolytes is the cement substance only. The third is to determine whether, as believed by Krogh (1937, 1946) the dissolved substances of the plasma pass the capillary wall "in bulk," *i.e.*, if all go across the capillary wall in the same concentration as exists in the plasma or whether these substances cross the wall in a proportion different from that maintained in the plasma. And finally it may be anticipated that experiments with the isotope-tracer technique will contribute to our understanding of the physical forces involved in transfer across the capillary wall. In those experiments which came before this method was available, investigators were forced to observe increments of fluid movement into or out of the capillaries (*cf.*, for example, the beautiful experiments of Landis, 1927, on single capillaries), were unable to resolve the components in one or the other direction of this net movement and could make only indirect conclusions about the movement of solutes other than dyes. Use of the tracer technique can consequently be anticipated to lead to some reorientation of viewpoint.

Our first problem was to measure the permeability of the walls of the capillaries to the substances of the blood plasma, *i.e.*, the rate at which these substances cross the walls of the vessels to reach the tissue fluids outside of the vessels. We began by measuring the rate of transcapillary exchange of water (Flexner, Gellhorn and Merrell, 1942) and sodium (Merrell, Gellhorn and Flexner, 1944) in the anesthetized guinea pig and have more recently added chloride (Cowie, Flexner and Wilde, 1948) and iron (Flexner, Vosburgh and Cowie, 1948). In its simplest form, our argument is that if we can experimentally determine the manner in which intravenously injected radiosodium, for example, leaves the plasma then we can from these observations calculate the rate for the normally occurring sodium of the plasma. What might we expect to happen in a qualitative way to the concentration of radiosodium after its intravenous injection? If the walls of the vessels are highly permeable to sodium, we would anticipate that immediately after injection the radiosodium would diminish rapidly in quantity in the plasma and its concentration would increase in the fluid outside of the vessels. As its concentration outside of the vessels increased, more and more would diffuse back into the vessels and the apparent rate of loss from the vessels would decrease until finally a steady state or equilibrium was reached in which the rate of loss from the vessels would equal the rate of return from the fluids outside the vessels. This is exactly what has been found experimentally in the guinea pig as shown in Figure 1. In its initial stages the concentration of radiosodium falls rapidly, then more slowly and in about 8 minutes reaches a

steady state which is maintained for the period of observation. The observations on the 10 animals of Fig. 1 were placed on a common basis by adjusting them all to the same initial concentration of radiosodium in the plasma (Merrell, Gellhorn and Flexner, 1944).

To derive the rate of movement of the normally occurring sodium of the plasma to the extravascular fluid it is assumed that the amount of radiosodium lost from the plasma per unit of time is proportionate to (1) the number of mg. of normally occurring sodium which moves from plasma to extravascular fluid per unit of time and (2) the proportion of the total sodium of the plasma which is radioactive. These assumptions can be expressed symbolically by stating that the loss in the total amount of radiosodium from the plasma with respect to time $= -r\frac{N_t}{Na_p}$, where r = total mg. of sodium which escape from the capillaries (or into the capillaries from the extravascular fluid) per unit of time; Na_p = total mg. of sodium in the plasma; N_o = quantity of radiosodium injected into the plasma at time, t = 0; and N_t = quantity of radiosodium in the plasma at any time after t = 0. Part of the radiosodium which escapes into the extravascular fluid will return to the plasma. The amount which returns per unit of time is proportionate to 1) the total amount of normally occurring sodium which moves from extravascular fluid to plasma per unit of time and 2) the proportion of the extravascular sodium which is radioactive. Expressed symbolically, the gain of radiosodium by the plasma from the extravascular fluid with respect to time will be equal to $r\frac{N_o - N_t}{Na_E}$ where Na_E = total mg. of sodium in the extravascular fluid. The net change in amount of radiosodium in the plasma with respect to time, $\frac{dN_t}{dt}$, will be given by the sum of these two processes:

$$\frac{dN_t}{dt} = -r\frac{N_t}{Na_p} + r\frac{N_0 - N_t}{Na_E} \qquad (1)$$

In terms of concentration of radiosodium in the plasma, equation (1) becomes:

$$\frac{dC_t}{dt} = -r\frac{C_t}{Na_p} + r\frac{C_0 - C_t}{Na_E} \qquad (2)$$

Integrating equation (2) and solving for the constant of integration give:

$$q[1n(C_t - C_{eq}) - 1n(C_0 - C_{eq})] = -Rt \qquad (3)$$

where $R = r/Na_p$ = the proportion of the plasma sodium which escapes from the plasma into the extravascular fluid per unit of time, $q = Na_E/(Na_E + Na_p)$ = the proportion of the total sodium which is extravascular, and C_{eq} = the concentration of radiosodium in the plasma at equilibrium.

Equation (3) states that the concentration of radiosodium in the plasma approaches equilibrium in such a way that the logarithm of the concentra-

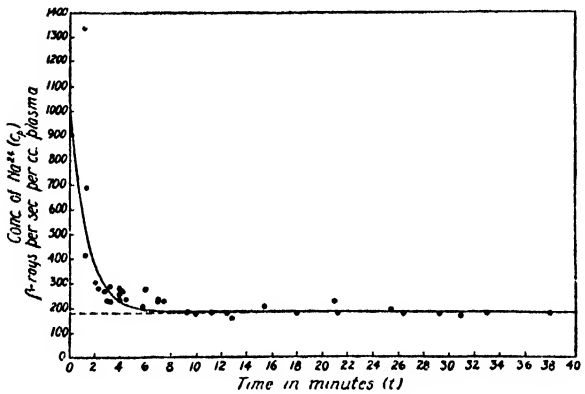

FIG. 1. Change in concentration of radiosodium in the plasma of the guinea pig with respect to time after intravenous injection. ($C_t - 181 = 819e^{-\frac{.80}{.819}t}$).

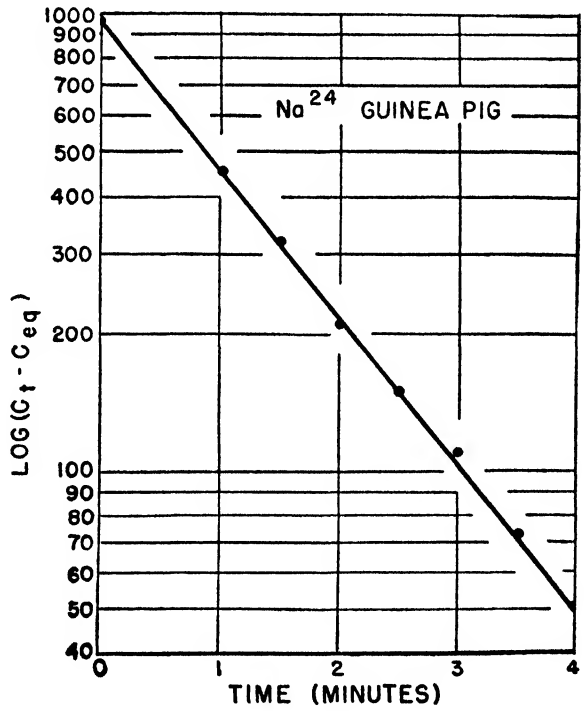

FIG. 2. Method of determining R/q from data of Fig. 1. The slope has been derived by dividing the decrement for any period in the log to the base 10 of $C_t - C_{eq}$ by the corresponding time in minutes, and multiplying by 2.3 to convert the log to the base e.

tion in excess of the equilibrium concentration is a linear function with time. In exponential form equation (3) may be expressed as

$$C_t - C_{eq} = (C_0 - C_{eq})e^{-Rt/q} \qquad (4)$$

which states that the excess concentration is reduced by a constant proportion per unit of time.

If this treatment is correct, we would expect when the logarithm of $(C_t - C_{eq})$ is plotted against time that the points would fall about a straight line the slope of which would equal $-R/q$. That this is so is evident from Figure 2. The value of R obtained from this slope and from the value of q (Merrell, Gellhorn and Flexner, 1944) is 0.60. This states that 60 percent of the sodium of the plasma leaves the plasma each minute to be replaced by sodium from

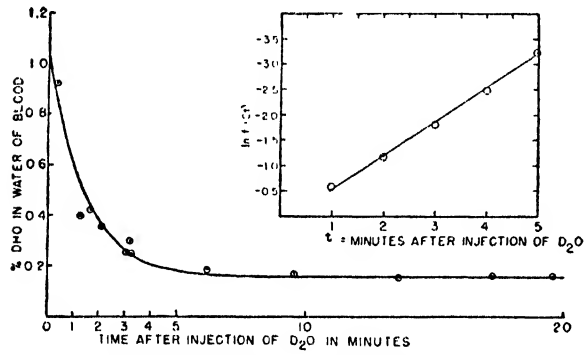

FIG. 3. Change in concentration of DHO in the water of the blood of the guinea pig with respect to time. Observations on 5 animals have been adjusted to a C_0 value of one gm. DHO per 100 ml. of water in blood. To convert the rate of turnover of water of whole blood to rate of turnover of water of plasma, the former value has been multiplied by by the ratio of water of whole blood to water of plasma (Merrell, Gellhorn and Flexner, 1944).

the extravascular source. We were greatly surprised by the rapidity of this process.

Even higher rates have been found for water (Flexner, Gellhorn and Merrell, 1942) and an equally rapid rate for chloride (Cowie, Flexner and Wilde, 1948). Deuterium oxide has been used as the tracer substance for water, radiochloride (Cl^{38}), for chloride. As is evident from Figure 3, the disappearance curve of DHO from whole blood is similar to that for radiosodium from plasma. The data, when analyzed as with radiosodium and when expressed in terms of the rate of turnover of plasma water rather than that of whole blood, show that 140 percent of the water of the plasma is exchanged with extravascular water per minute. The data with radiochloride are shown in Figure 4. From these it can be shown that 60 percent of the chloride of the plasma is exchanged each minute with extravascular chloride. The measurements and calculations which have been reviewed show that the vascular wall of the guinea pig is more permeable to water than to chloride and equally permeable to chloride and to sodium. Since the water of the plasma is turned over at the rate of 140 percent per minute and the sodium and chloride at the rate of 60 percent we can say with considerable precision that the capillaries of the guinea pig are on the average 2.3 times as permeable to water as to sodium and chloride.

The behavior of iron is considerably different (Flexner, Vosburgh and Cowie, 1948). Iron in the plasma, as shown by the work of E. J. Cohn and his group (1947), is present as ferric beta$_1$-globulinate. We have obtained this substance, tagged with radioiron, from a donor animal. Radioiron was given by stomach tube to an anemic guinea pig and the animal was bled after sufficient time had been given for the radioiron to appear in its plasma (Hahn *et al.*, 1939). This plasma containing ferric beta$_1$-globulinate tagged with radioiron was then transfused into a recipient. Samples of plasma were taken for measurement of their radioactivities up to 150 minutes after transfusion. Since there is no evidence during this interval of return to the plasma from the extravascular fluid of radioiron lost from the plasma,

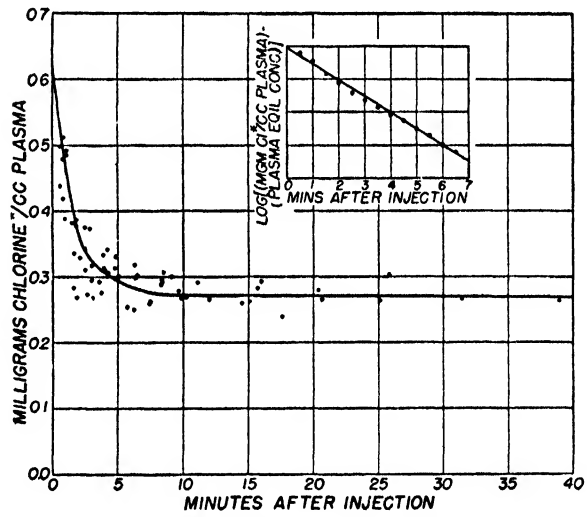

FIG. 4. Change in concentration of radiochloride in the plasma of the guinea pig with respect to time. Observations on 8 animals have been adjusted to the same C_0 value. Since chloride of plasma comes into equilibrium with the chloride of red cells with great rapidity and the chloride of red cells is 50 percent that of plasma, the apparent rate of turnover of plasma chloride must be multiplied by 1.5 to give the true rate.

it can be anticipated, on the basis of the mathematical treatment of the data with radiosodium given above, that the change in concentration of radioiron in the plasma with respect to time will be given by the equation:

$$\ln C_t = -Rt + \ln C_0 \qquad (5)$$

R = proportion of the iron of the plasma which escapes from the plasma into the extravascular fluid per unit of time and C_t and C_o are respectively the concentrations of radioiron at any time, t, and at t = 0. As predicated by equation 5 and shown in Figure 5 when the logarithm of the concentration of radioiron in any experiment is plotted against time the points fall about a straight line the slope of which is equal to R, the proportion of the iron which escapes from the plasma per unit of time.

Considerable variation in the rate at which iron is lost from the capillaries is evident among the 8 animals. In the guinea pig with minimal rate, only 0.3 percent of that present was lost per minute; in the guinea pig with maximal rate, 1 percent was lost per minute. Expressed in other terms, one-half of the iron present at any instant was lost from the plasma in 288 minutes in the first of these animals and in 63 minutes in the other.

The contrast between the permeability of the capillaries to sodium, chloride and water on the one hand, and iron on the other is a striking one. In the guinea pig, 60 percent of the sodium and chloride and 140 percent of the water of the plasma is exchanged each minute with extravascular sodium and water. Only from 0.3 to 1 percent of the ferric globulinate is lost from the plasma per minute. If the capillaries were equally permeable to all these substances, their rates of turnover in the plasma would be identical. The results permit us to say that the capillaries are at least 100 times as permeable to water as to iron. On the assumption that iron crosses the capillary wall as the globulinate and is not split from this molecule within the endothelial cells, the fluid passing through the capillary wall contains on the average 1 percent or less of the ferric globulinate of the plasma and fits Landis' (1934) statement "that the capillary endothelium can, and often does, retain 95 percent of the plasma protein." We are currently endeavoring to distinguish possible differences in the permeability to iron of the capillaries of various organs of the guinea pig by measuring the rate at which iron from the plasma appears in them.

During the war, this type of approach to determination of the rate of transcapillary exchange was used in our laboratory in a study of shock by Gellhorn, Merrell and Rankin (1944). The experimental animal was the dog. We shall consider here briefly only that part of their work concerned with the rate of transcapillary exchange of sodium in the normal animal. From the viewpoint of this presentation their important observations were as follows: 1) the decline in concentration of radiosodium after intravenous injection dose not involve a single rate as in the guinea pig but two rates are necessary and sufficient to describe the process, and 2) Gellhorn, Merrell and Rankin obtained evidence of a kind previously noted by Manery and Bale (1941) and Hahn and Hevesy (1941) that the structures of the body can be divided into two groups, A and B, on the basis of the rate of transcapillary exchange of sodium within them. Group A, comprised in part of muscle, lung, intestine and liver, is characterized by a relatively rapid rate of transfer of sodium between plasma and extravascular fluid. Group B containing skin, tendon, bone and brain is characterized by a relatively slow rate of transfer between plasma and extravascular fluid. Gellhorn, Merrell and Rankin concluded that the two rates necessary to describe the decrease in concentration of radio-

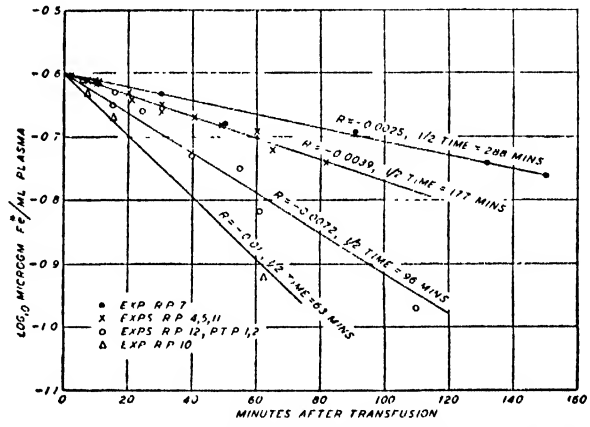

FIG. 5. Change in concentration of labeled ferric beta$_1$-globulinate in the plasma with respect to time after intravascular injection. R has been derived by dividing the decrement for any period in the log to the base 10 of the concentration of radioiron by the corresponding time in minutes and multiplying by 2.3 to convert the log to the base e.

sodium after intravenous injection in the dog are due to unequal rates of transfer across the capillary bed in different parts of the body. It is obvious, as they say, that in any system as involved as the capillaries, such a hypothesis is an oversimplification, but it serves to describe the major effects.

This work has been of great aid to us in our efforts to determine the rate of transcapillary exchange of sodium and water in man, for in man, as in the dog, the disappearance of the tracer substances from the plasma is described by a double exponential. The data on man, however, present a considerable difficulty not encountered in the guinea pig or dog. In the guinea pig and dog mixing of the tracer substance with the plasma is essentially complete in one minute. We have attempted to estimate mixing time in our three human subjects (all of whom were at intermediate stages of pregnancy) by taking simultaneously, from veins of both arms, samples of blood in the earlier parts of the experiments with radiosodium. It was assumed that mixing was essentially complete when the amount of radiosodium in samples taken simultaneously from the two arms was about equal. As can be seen in Figure 6, mixing time estimated in this way varied from 8 to 13 minutes. As is also evident in the figure, by the

time that mixing was complete, the concentration of radiosodium had decreased greatly from its initial value and was relatively near the concentration at "equilibrium." The concentrations from 2 to 3.5 to 3.5 minutes fall about a straight line which at t = 0 gives the anticipated initial concentration of radiosodium. It is therefore evident that the curve of disappearance of radiosodium from human plasma

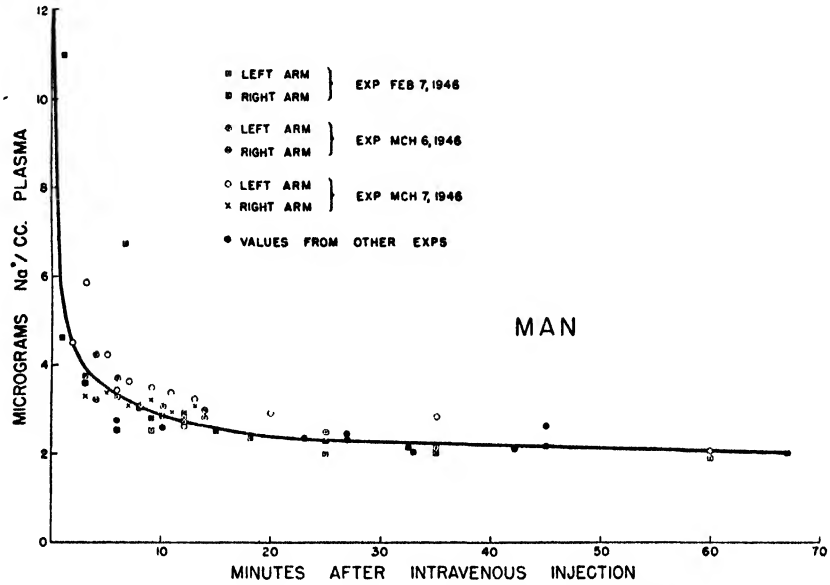

FIG. 6. Change in concentration of radiosodium in the plasma with respect to time. The points from the 3 pregnant subjects were adjusted to a standard basis by multiplying them all by a factor which brought the initial concentration to 12 microgm. per ml. of plasma.

minutes after injection as well as later values are essential for determining the rate at which plasma sodium crosses the capillary wall (Figure 7). Our only means of meeting the difficulty of incomplete mixing is to assume that the average of the concentrations after 2 minutes in the two arms is for our purposes not significantly different than the value which would have been obtained had mixing been rapidly completed. Our justification, which cannot be considered complete, for this procedure is twofold: 1) the average concentrations at 5 minutes and up to the time of complete mixing fall, as will be shown, on the same logarithmic curve as the values obtained after complete mixing and 2) the average values from 2 to 3.5 minutes yield on extrapolation the concentration of radiosodium at t = 0 calculated from the known amount injected and the plasma volume of the subject.

We have used graphic methods for analysis of the data. The curve of Figure 6 has been fitted to the observations by inspection. The plot of the logarithm of $C_t - C_{eq}$ is given in Figure 7. It is apparent that more than a single rate is involved since this is not a straight line. The portion of the curve from 5 to 16 minutes, however, is linear. Whether two or more rates are present is then tested by subtracting the values of the extrapolated portion of the curve B_2 from the curvilinear portion of the plot of logarithm $(C_t - C_{eq})$. Values obtained in this way from 2 involves two rates and can be described by the equation used for the dog:

$$C_t - C_{eq} = a_1 e^{b_1 t} + a_2 e^{b_2 t} \qquad (6)$$

The constants of equation (6) are obtained from

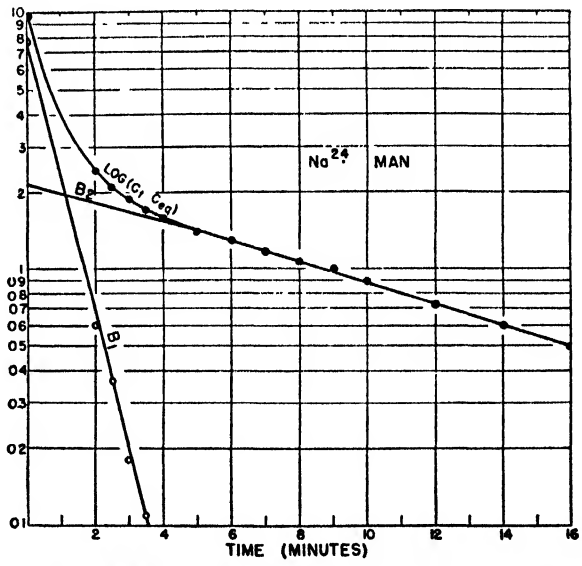

FIG. 7. Method of evaluating the constants of equation (6) for sodium. Details of the method are given in the text.

Figure 7. The values of a_1 and a_2 are given respectively by the intercepts at t = 0 of lines B_1 and B_2. The rate constants b_1 and b_2, which must be evaluated in terms of the natural logarithm, are obtained by multiplying the slopes of B_1 and B_2 by 2.3. Equation (6) consequently becomes:

$$C_t - 2.2 = 7.6e^{-1.2t} + 2.2e^{-0.090t} \qquad (7)$$

The concentrations of radiosodium as a function of time after intravenous injection calculated from this equation agree closely with the observed average values described by the curve of Figure 6.

The constants of this equation are then substituted in equations 4 to 12 of Gellhorn, Merrell and Rankin to determine the rate of turnover of the sodium of human plasma. We find in this way that

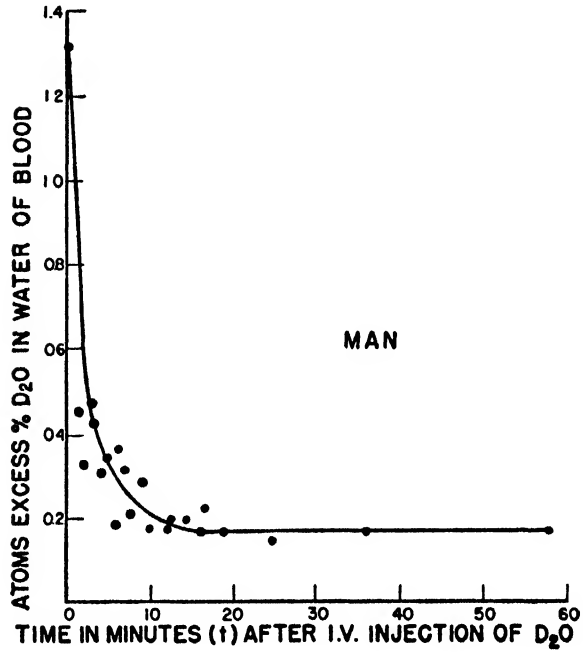

FIG. 8. Change in concentration of DHO in the whole blood with respect to time. The observations from the 3 pregnant subjects have been adjusted to a common basis.

78 percent of the plasma sodium in our pregnant human subjects is exchanged per minute with extravascular sodium. Area A receives 64 percent of the plasma sodium per minute; area B, 14 percent. Burch, Reaser and Cronvich (1947), using the same experimental method, reported that 32 percent of the plasma sodium was exchanged per minute in a normal human subject.

Figure 8 shows the results in man obtained with D_2O. Here again, as might be anticipated, we encountered the difficulty that thorough mixing was not obtained prior to completion of the first rapid rate of disappearance which could be analyzed only for the first 2.5 minutes of the experiment. The data have been analyzed like those obtained with radiosodium. Two rates are necessary and sufficient to describe the process as shown in Figure 9. We find, using the same procedures as with sodium, that 105 percent of the plasma water is exchanged per minute

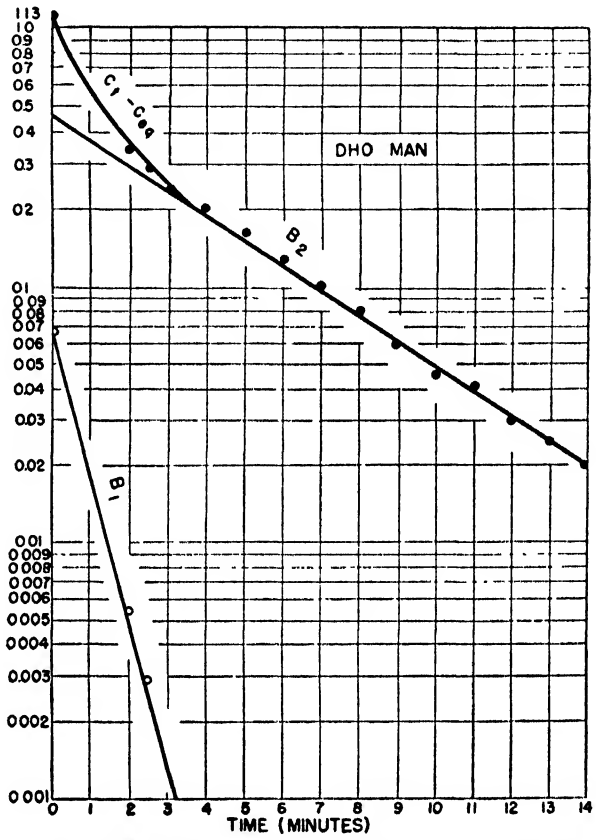

FIG. 9. Method for evaluating the constants of equation (6) for water. To obtain the rate of turnover of plasma water from that of whole blood, the latter has been multiplied by 1.42, the ratio of total blood water in man to plasma water (Gibson, Peacock, *et al.*, 1946; Peters and Van Slyke, 1931).

with extravascular water. Area A receives 63 percent of the plasma water per minute, area B, 42 percent.

We can consequently say in man as in the guinea pig that the total capillary bed is more permeable to water than to sodium. The situation in man gains added interest because we have an opportunity not only to compare the overall rates but in addition to estimate the rate of escape into two groups of organs characterized by differences in the apparent behavior of their capillary beds. In the first of these, characterized by a rapid rate of exchange between plasma and extravascular fluid, water and sodium appear to penetrate with equal rapidity. This conclusion, as has been stressed, is weakened by inadequacy of mixing and the short duration of this rate. In the

second group of organs, the capillaries appear to be three times as permeable to water as to sodium. This difference can be accepted with assurance since the rates both for water and sodium have been established after mixing is complete.

Our experience with iron in man is limited. Vosburgh (1948) has measured the rate of disappearance of ferric beta$_1$-globulinate from the plasma of several normal subjects with results closely like those obtained on the guinea pig. Figure 10 gives the data of a single experiment in which 0.7 percent of the ferric globulinate is lost per minute from the

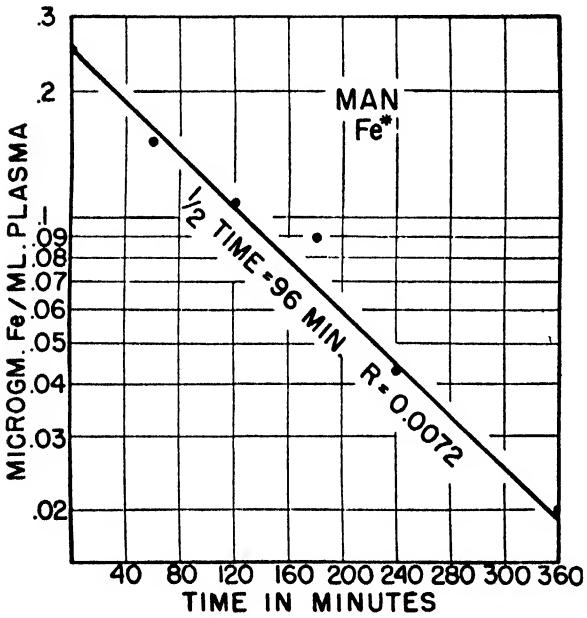

FIG. 10. Change in concentration of labeled ferric beta$_1$-globulinate in plasma of a normal subject with respect to time after intravenous injection (Vosburgh, 1948).

plasma. It is evident that in man as in the guinea pig the capillaries on the average are at least 100 times as permeable to water as to the globulinate.

DISCUSSION

As has been said, we were greatly surprised by the rapidity of exchange, between the vascular system and the extravascular fluid, of water and of those electrolytes which we have studied. In terms used by Burch, Reaser and Cronvich (1947), it can readily be shown that water in a quantity amounting to the weight of the animal or man is lost each 20 minutes from the plasma to be replaced by water from the extravascular fluid. The rate of turnover of plasma sodium and chloride is hardly less spectacular but the weight of these substances moved from plasma to extravascular fluid is of course far less than in the case of water due to their low concentrations in the plasma. Per day, however, a quantity of sodium chloride approximately equal to 30 percent of the weight of the animal or man moves from plasma to extravascular fluid. In the light of the great quantity of fluid continuously being transferred between plasma and extravascular fluid, it can be appreciated that the accumulation of extravascular fluid which gives rise to edema represents only a slight, though important deviation from the normal.

We come now to the interesting problem proposed by Chambers and Zweifach (1940, 1947) as to the portion of the capillary wall through which electrolytes are exchanged. We believe that it is possible to show that the whole wall of the capillary or certainly the endothelial cell must be permeable to chloride, for example, rather than the intercellular cement alone or in major part. We shall assume, following Krogh (1922), that the average diameter of all the functional capillaries of the body at rest is the same as the average diameter of a red blood cell. The average diameter of a red blood cell of the guinea pig is given by Krogh (1922) as 7.2 micra. This means that 1 ml. of plasma in the capillaries is exposed to an area of capillary wall of 5600 cm.^{2}. How much chloride would diffuse across this area per minute if the wall offered no resistance to its movement? We have chosen for this calculation to use the diffusion constant for KCl since anion and cation rates of diffusion are closely alike. The diffusion constant for 0.01 N KCl at 38° C is 2.15 cm.^{2} per day. This constant when multiplied by the area of interface and the concentration gradient expressed in grams or equivalents per ml. of solution (Clack, 1923) will give the maximum amount of chloride which can diffuse across the interface per day. Each ml. of plasma contains 0.1 millieq. of chloride. This concentration of chloride, 0.1 millieq. per ml., is for our purposes the effective concentration, since the data on the rate of disappearance of radiochloride have been analyzed to give total quantity of chloride which moves per unit of time from plasma to extravascular fluid and not a difference between that moving in two directions. The maximum amount of chloride which would diffuse from 1 ml. of plasma across an interface of 5600 cm.^{2} per minute is consequently $2.15 \times 5600/1440 \times 10 = 0.86$ millieq. This quantity is now to be compared to the amount which has been observed to move across the capillary wall. It has been observed (Cowie, Flexner and Wilde, 1948) that 60 percent of the plasma chloride of the guinea pig is exchanged each minute with extravascular chloride. As shown by the work of Gibson, Seligman, *et al.*, (1946), however, only 20 percent of the plasma is at any intant in the minute vessels so that 60 percent of 5 ml. of plasma is exchanged per minute across 5600 cm.^{2} of wall, *i.e.*, 5×0.06 millieq. $= 0.3$ millieq. If, as assumed by Chambers and Zweifach (1947), 1 percent of the capillary wall is intercellular cement and if the intercellular cement alone were permeable to chloride, only .0086 millieq. could diffuse per minute. This is a quantity less than 3 percent of that ob-

served. It could be objected that diffusion may not be the sole process in transport of chloride from plasma to extravascular fluid and that filtration obscures the meaning of these calculations. The quantity of chloride which moves across the capillary membrane from plasma to extravascular fluid is, however, at least approximately equal to that moving from extravascular fluid to plasma and this latter process is considered to be purely diffusion (Starling, 1895). It is clear, therefore, that the total capillary wall is likely freely permeable to chloride and the same deduction can be made for water and sodium.

The evidence at hand, while to date admittedly limited with respect to the number of substances which have been examined, indicates strongly that water and solutes do not cross all parts of the capillary bed in bulk, *i.e.*, in the same proportion as they exist in the plasma. For this condition to be satisfied, the rates of turnover of plasma water and sodium would be identical. This may be so in one part of the capillary bed of man but caution must be used in evaluation of these results due to incomplete mixing of the tracer with the plasma and the short duration of the rate which is involved. The results are without this objection in the guinea pig and in a second portion of the capillary bed of man

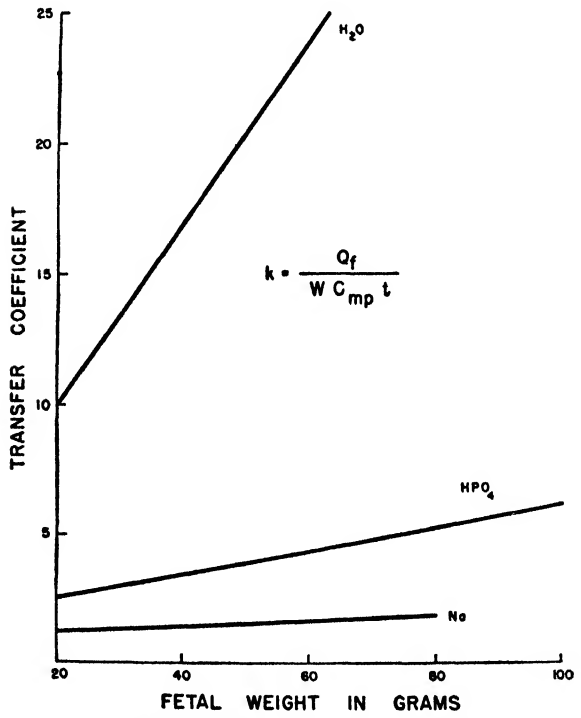

FIG. 11. Placental transfer coefficients for sodium, inorganic phosphate and water at various fetal weights (guinea pig). The coefficient k has been derived by referring the quantity of substance transferred to the fetus (Q_f) from the maternal plasma to unit weight of placenta (W), unit concentration of the substance in the maternal blood (C_{mp}) and unit time (t).

where the capillaries appear to be two to three times as permeable to water as to sodium. Evidence along these lines was obtained by Hahn and Hevesy (1941). They concluded that potassium crosses the capillary wall at a much faster rate than any other ion which they investigated. Several examples may be given of mammalian membranes more complex

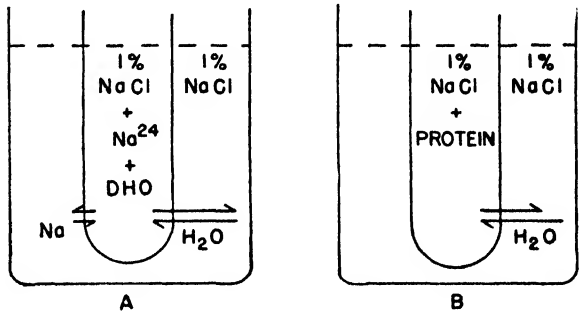

FIG. 12. Diagrammatic representation of the movement of water and sodium ions across a celloidin membrane as described in the text. "A" indicates difference in permeability of the membrane to H_2O and sodium at equilibrium as demonstrated by the tracer technique. "B" indicates the effect of adding protein, to which the membrane is impermeable, on the rate of escape of water from inside the sac. This rate is lowered while the rate of movement of water in the opposite direction is unaffected.

than the capillary wall where the differences among the apparent permeabilities to water and ions are greater than in the case of the capillary. As shown in Figure 11 (Wilde, Cowie and Flexner, 1946), the apparent permeability of the placenta to water, depending on the gestation age, is from 10 to 16 times that for sodium and the permeability to inorganic phosphate is two to three times that for sodium. The same sort of results have been obtained on amniotic fluid (Flexner and Gellhorn, 1942) and on the aqueous humor of the eye (Kinsey, Grant, *et. al.*, 1942; Wilde, Scholz and Cowie, 1947).

These results, showing that the wall of a capillary may be more permeable to water than to sodium or chloride, have been of greatest interest to us because they reveal something of the physical process by which water and solutes pass from the plasma to the extravascular fluid. This process cannot consist entirely of ultrafiltration through pores for it has been shown (Flexner, 1934; Flexner, 1937) that capillary hydrostatic pressure is too low to effect by filtration significant separation of solvent from solute. If ultrafiltration accounted completely for the separation of fluid from the plasma, the rates of turnover of all the constitutents of the filtrate except protein would be the same. Diffusion through a membrane can, however, account for differences of the kind observed. To demonstrate this we (Gellhorn and Flexner, 1942) have performed a simple experiment with a celloidin sac. The sac was immersed in and filled with a 1 percent solution

of NaCl (Figure 12A). To measure the relative rates of movement of water and sodium across the membrane under this equilibrium state, we added tracer quantities of radiosodium chloride in D_2O to the inside of the membrane. Radiosodium and deuterium appeared in the fluid outside the membrane at a rate which showed that the membrane was eight times as permeable to water as to sodium.

What proportion of the total exchange of water, for example, between plasma and extravascular fluid can be accounted for by diffusion? We shall consider a capillary in a tissue at rest where lymph formation is negligible so that we are assured that the rate of escape of water from the plasma is equal to the rate at which it passes back into the plasma from the extravascular fluid. Following Starling (1895) and subsequent authors, we assume that substances pass from the extravascular fluid to the plasma by diffusion only. The amount of water diffusing across the capillary wall per unit time is proportional to the thermodynamic activity (Glasstone, Laidler and Eyring, 1941) of the water (escaping tendency or effective concentration rather than actual concentration). In the presence of colloidal protein, the activity of water is lowered and as shown in Figure 12B when protein is added to a celloidin sac filled with and immersed in 1 percent NaCl, the rate of movement of water out of the sac is diminished, the rate of movement into the sac remaining unchanged. How much does protein in a concentration which exerts an osmotic pressure of 0.024 atmospheres, as in the guinea pig (Landis, 1934), lower the activity of water? The activity of water under these conditions can be shown by thermodynamics (Flexner, 1934) to be lowered about 0.02 percent. This means that while 5000 grams of water pass into the celloidin sac from the protein-free medium surrounding it, 4999 grams pass from the protein containing solution within the sac to the fluid outside it. It we consider a capillary instead of the celloidin sac, the missing gram of water in the 5000 is accounted for by hydrostatic pressure which raises the activity of water sufficiently for its escape.

This argument, based on the assumption that substances return to the plasma from the extravascular fluid by diffusion and predicating capillaries in a tissue where lymph-formation is negligible, represents a considerable departure from the interpretation often given the Starling hypothesis. This hypothesis, as frequently presented, emphasizes the importance of separation of fluid by filtration at the arteriolar end of the capillaries and reabsorption of fluid by "osmotic attraction" at the venular end of the capillary. Diffusion, while not neglected, often is relegated to a minor role. It is our view that in at least many of the capillaries of the body diffusion is the essential process by which exchange of water and solutes takes place between plasma and extravascular fluid. The relationship between colloid osmotic pressure and capillary pressure, the central feature of the Starling hypothesis, becomes important in capillaries such as these when the rapid rate of exchange of water is considered. One gram of water in 5000 becomes obviously significant when related to the high rate of turnover, approximately once every 40 seconds, of the water of the plasma. The potential deficit due to the very slightly lowered activity of plasma water by plasma proteins is corrected by the increase in activity brought about by the hydrostatic pressure of the capillary. This tentative concept of the relationship among diffusion, the Starling hypothesis and the rate of turnover of the substances of the plasma is an important gain which has come to us from using tracer substances to investigate capillary permeability. It is evident that we must put our views to further test by measuring the rates of transcapillary exchange of other constituents of the plasma.

Summary

The rates of turnover of plasma water, sodium, chloride and ferric beta$_1$-globulinate have been determined in the guinea pig by the isotope-tracer technique. 140 percent of plasma water is exchanged each minute with extravascular water, 60 percent of plasma sodium and chloride is exchanged each minute while only from 0.3 to 1 percent of the ferric beta$_1$-globulinate is lost per minute from the plasma. The results show that the average capillary wall of the guinea pig is 2.3 times as permeable to water as to sodium and chloride and at least 100 times as permeable to water as to ferric beta$_1$-globulinate.

In man, 78 percent of the plasma sodium and 105 percent of the plasma water is exchanged per minute with extravascular sodium and water.

On the basis of Fick's law of diffusion and the rate of transcapillary exchange of chloride, it is shown that the whole wall of the capillary or certainly the endothelial cell must be permeable to chloride rather than the intercellular cement alone or in major part. The same deduction can be made for water and sodium.

Diffusion rather than filtration is concluded to be the essential process in the exchange of substances across the walls of at least many of the capillaries of the body at rest, and in these accounts for all but about 0.02 percent of the total water which is exchanged. In these capillaries the forces involved in Starling's hypothesis become clearly important when the high rate of transcapillary exchange of water is considered.

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DISCUSSION

NEUMAN: I think a reasonable objection may be raised to the use of radioiron as a label for the rate of exchange of blood globulin. It must first be established that iron, bound by globulin, does not undergo dissociation. This would be very simple to test in a system *in vitro*. With the data at hand such a dissociation is suggested, and I would agree with Dr. Michaelis that, in view of the extremely small quantities of free ionic iron present in plasma, the equilibration between the ferric ions in plasma and extracellular fluid may be very rapid indeed, quite comparable to that observed for sodium and chloride.

FLEXNER: We agree that it has not been demonstrated beyond doubt that radioiron may serve as a label for plasma globulin. Dr. Vosburgh, however, has brought to my attention evidence which supports this hypothesis. In his excellent and notably comprehensive monograph on serum iron, Carl-Bertil Laurell records the following experiment. A semipermeable membrane filled with human serum, enriched with iron, was immersed in serum and dialysis continued for 24 hours at five degrees centigrade. The iron content of the serum within the membrane was determined before and after dialysis. No iron could be demonstrated to have crossed the membrane until the iron-binding capacity of the serum within the membrane had been exceeded. It is possible that the same experiment with radioiron may give a more sensitive test and we plan, following Dr. Neuman's suggestion, to try this. It must also be noted that iron may be split from globulin within the endothelial cells of the capillaries. This possibility can perhaps be explored by comparing the rate of disappearance of tagged globulin from the plasma with that of radioiron.

HEVESY: Dr. Flexner described most interesting experiments in which he injected plasma containing labeled iron into the circulation. May I ask

Dr. Flexner if he has any experience to show at what rate labeled iron introduced into the circulation penetrates through the capillary wall?

FLEXNER: We have a few experiments in which labeled iron was injected intravenously as ferric chloride and its plasma concentration measured from 3 to 160 minutes after the injection. These disappearance curves are described by a double exponential. One rate, which can be evaluated from 30 minutes after the injection to the end of the experiment, is typical of labeled ferric beta$_1$-globulinate and is ascribed to the disappearance of this substance from the plasma. A considerably greater rate of disappearance of labeled iron is evident during the first 30 minutes of the experiment and we believe this to be due to the disappearance of labeled iron not bound to beta$_1$-globulin *i.e.* to iron which may possibly be present as the ascorbate or hydroxide or loosely bound to plasma protein. This iron disappears at the rate of about 25 percent of that present per minute and is to be compared to a rate of from 0.3 to 1 percent for the globulinate. These observations have recently appeared in the Amer. J. Physiol. *153:* 503, 1948.

ISOTOPES IN PHARMACODYNAMICS

CHALMERS L. GEMMILL

Pharmacodynamics may be defined as that branch of Pharmacology which deals (1) with the action of drugs on living organisms, (2) with the absorption of drugs and their fate in the body and (3) with the correlation of drug action and chemical constitution (Goodman and Gilman, 1941). This definition will be used to limit this review to the application of tracer elements to the explanation of drug action on living organisms, and to the tracing of drugs through the animal body. Therefore, very little will be said of the therapeutic use of tracer substances or the toxicology of radioactive elements. Pharmacologists have not made as much use of tracer elements as biochemists and physiologists. In fact, in the past four volumes of the *Journal of Pharmacology and Experimental Therapeutics* there is only one article in which a tracer substance has been used.

In Table 1 is given a list of tracer elements which have been employed to explain drug action. In this review, examples will be given of the use of each of these tagged elements in pharmacodynamics.

Hydrogen 3 and Carbon 14. Phenylalanine has been prepared labeled with tritium and the same compound tagged with carbon 14 in the carboxyl group and in the α-carbon (Gurin and Delluva, 1947). It was shown that these compounds can be converted into adrenalin by the animal body. The authors conclude that the side chain of phenylalanine remains attached to the benzene ring during the conversion of phenylalanine to adrenalin. The experiments illustrate the method of biological production of a pharmacologically active substance.

Radioactive carbon in carbon monoxide has been used in the study of elimination of this toxic gas (Tobias, Lawrence, Roughton, Root and Gregersen, 1945). In this study normal men were given radioactive carbon monoxide in a breathing mixture. Examination of the expired carbon dioxide revealed that very little of the carbon monoxide was oxidized to carbon dioxide in the human body.

Another use of radioactive carbon has been in the study of the metabolism of 1:2:5:6 dibenzanthracene labeled in the 9-position with carbon 14 (Heidelberger and Jones, 1947). It was shown that when this carcinogenetic substance was injected intravenously as a colloid, large quantities were eliminated in the feces. When subcutaneous injections were made, 52 percent of the dibenzanthracene remained near the site of injection. In a tumor which appeared at the site of injection, some of the dibenzanthracene was found to be converted to an acidic form and another fraction to a phenolic form.

Two compounds have been prepared containing carbon 14 which are of pharmacological interest: urethan (Skipper, Bryan and Hutchison, 1947) and testosterone (Turner, 1947). To the author's knowledge, these two substances have not been used in pharmacological experimentation.

Nitrogen 15. Sodium pentobarbital has been prepared containing nitrogen 15 (Van Dyke, Scudi and Tabern, 1947). When this compound was fed to dogs, only a small fraction of the tagged nitrogen was found in the urinary ammonia and urea. Ninety-two percent was excreted as pentobarbital or metabolic degradation products derived from the drug. It was shown also that the degradation products contained the greater part of the excreted nitrogen.

Table 1. Tracer Elements Used in Pharmacodynamics

Element	Atomic number	Atomic weight
Hydrogen	1	3
Carbon	6	14
Nitrogen	7	15
Oxygen	8	18
Fluorine	9	18
Sodium	11	24
Phosphorus	15	32
Sulfur	16	35
Calcium	20	45
Manganese	25	52 and 56
Iron	26	55 and 59
Copper	29	64
Arsenic	33	74
Selenium	34	75
Bromine	35	82
Strontium	38	89
Antimony	51	122 and 124
Iodine	53	131
Gold	79	198
Mercury	80	197
Lead	82	210

Trimethylamine and acetylcholine labeled with nitrogen 15 have been used in a study of the diffusion of these substances into the giant axons of the squid (Rothenberg, Sprinson, Nachmansohn, 1948). It was shown that the trimethylamine penetrates into the interior while acetylcholine is unable to diffuse into the axon. The authors use the difference in the diffusion of these two substances to explain some of the pharmacological actions of acetylcholine.

Oxygen 18. Oxygen 18 has been used in an investigation of the chemical formation of ether (Lauder and Green, 1946). However, the ether containing heavy oxygen has not been employed for pharmacological study.

Fluorine 18. The adsorption of radioactive fluorine in the form of sodium fluoride has been studied

in samples of enamel, dentin and bone (Volker, Hodge, Wilson and Van Voorhis, 1940). It was shown that fluorine can be adsorbed by these substances. These authors speculate that the adsorption of fluoride by enamel may be significant clinically, for the local application of solutions of fluorides to the teeth may add fluoride to the enamel surface in order to decrease the susceptibility to dental caries.

Sodium 24. Radioactive sodium has been used in a study of the efficiency of drugs used in treatment of disease of the peripheral vascular system (Mufson, Quimby and Smith, 1948). In patients suffering with various vascular system diseases, histamine increased the diffusion of radioactive sodium from the blood vessels when the histamine was administered by iontophoresis or by interarterial injection. Neither papaverine nor sodium chloride produced any change in the diffusion rate of radioactive sodium from the blood vessels into the tissues.

Sodium 24 has been used in the form of physiological solution of sodium chloride as a tracer to determine the deposition of aerosols within the respiratory tract of man (Talbot, Quimby and Barach, 1947). It was found that the "chest count" went up immediately after inhalation of the aerosol, decreased rapidly after the first one-half hour and more slowly for about three hours. The authors suggest that this method may be used to determine the efficiency of aerosol nebulizers.

Radioactive sodium has been employed in the form of sodium chloride to study the intake of this salt from enteric coated capsules (Lark-Horovitz, 1940). It was possible to test whether the capsule dissolves in the stomach, develops a leak or breaks in the small intestine.

Phosphorus 32. In spite of the widespread use of phosphorus 32 in biological tracer experimentation, only a few applications of this tagged element have been made in pharmacodynamics. The distribution of phosphorus has been traced following the injection of insulin. Insulin accelerates the rate of disappearance of phosphorus 32 from the blood and causes an increase in total acid soluble phosphorus 32 in the liver and muscle (Kaplan and Greenberg, 1944). These results indicate that insulin accelerates the transfer of inorganic phosphate from the blood into the muscle and liver. In resting cats, given glucose, insulin accelerates the turnover rates in the muscle of phosphocreatine and the two labile phosphate groups of adenosine triphosphate (Sacks, 1945). The distribution of phosphorus has also been studied following the administration of parathyroid extract (Tweedy, Chilcote and Patras, 1947). Parathyroid extract causes a prompt urinary excretion of administered radiophosphorus in the thyroparathyroidectomized rat. It was concluded that parathyroid extract has a direct action on the kidney.

Radioactive phosphorus in the form of phosphate is being used to study the uptake of phosphate by the red blood cell under the influence of various anesthetics (Pertzoff and Gemmill, 1948). It has been found that ether and sodium barbital inhibit the uptake of phosphate by the red blood cell after incubation at 37° C. for several hours.

Sulfur 35. There have been several applications of the use of radioactive sulfur in pharmacodynamics, Mustard gas labeled with radioactive sulfur is fixed in the epidermis and corium in human skin (Axelrod and Hamilton, 1947). The epidermal concentration is greater than that in the corium.

Radioactive sulfur was used also in the proof of the structure of synthetic penicillin (du Vigneaud, *et al.*, 1946). Estrone sulfate has been prepared labeled with sulfur 35 (1948).

Calcium 45 and Strontium 89. Normal and rachitic rats were given radioactive calcium 45 and strontium 89 in the form of lactate salts (Greenberg, 1945). The distribution of these two elements was followed both in vitamin D treated and untreated animals. It was shown that vitamin D promotes the absorption of calcium from the digestive tract and may have a direct effect on the mineralization of bone in rachitic rats.

Manganese 52 and 56. The distribution of radioactive manganese has been studied in perosis in chicks (Mohamed and Greenberg, 1943). This disease was produced by a synthetic manganese deficient diet. These authors demonstrated that deficient chicks retained a greater amount of manganese than the normal controls, but both groups excreted the major portion of the administered dose. Measurable amounts of radioactive manganese appeared only in the bone of the deficient chicks following injection of this substance. The liver contained the greatest amount of this substance.

It has been shown that manganese 52 administered to human subjects concentrates in the liver, with an intermediate concentration in pancreas and kidney (Sheppard, Wells, Hahn and Goodell, 1947).

Iron 55 and 59. Much of the work which has been done on the metabolism of iron using radioactive iron 55 or 59 has a bearing on the function of iron in pharmacodynamics. This subject has recently been reviewed (Sacks, 1948) and, therefore, will not be covered again in this paper.

Copper 64. The effect of radioactive copper has been studied on copper deficient rats (Schultze and Simmons, 1942). It was demonstrated that the copper deficient rats retained more copper than iron deficient animals. The kidneys, liver and bone marrow had the highest concentration of this element of any of the organs examined 24 to 48 hours after administration of the copper.

Arsenic 74. Interesting experiments have been carried out (Lawton, Ness, Brady and Cowie, 1945) by injecting sodium arsenite into cotton rats infected with *Litomosoides carinii.* It was found 24 hours after the injection that the filarides had a higher content of radioactive arsenic than any of 12 tissues

of the host. The authors conclude that the adult form shows a specific affinity for arsenic. The liver, kidney, skin, spleen and lung of the cotton rats have a high affinity for the radioactive arsenic. Also, studies of the distribution of radioactive arsenic injected as potassium arsenite have been made (Hunter, Kip and Irvine, 1942). It was found in the rat that arsenic is concentrated in the erythrocytes. In other animals, the greatest amount is found in skeletal muscles. In a second paper (Lowry, Hunter, Kip and Irvine, 1942), the authors describe the arsenic as being bound to the protein fraction of various tissues.

Two experiments have been carried out on human skin with lewisite labeled with arsenic 74 (Axelrod and Hamilton, 1947). The radioautographs showed that the arsenic was concentrated in the epidermis with very little in the dermis.

Selenium 75. Sodium selenate containing radioselenium has been used to study the distribution and excretion of this compound in rats. After a single subcutaneous injection (McConnell, 1941), the greatest concentration was found in the liver, while lesser concentrations were found in muscle, gastrointestinal tract and blood. The concentrations depended on the time of examination. For example, after the first half hour of injection, the greatest concentration appeared in the plasma, while after the third hour, the concentration in the red blood cells exceeded that in the plasma. Selenium was found to be excreted mainly by the kidneys, and to a lesser extent, by the gastrointestinal tract.

Bromine 82. Only a few uses of radioactive bromine have appeared in the literature. Radioactive bromine has been shown to be concentrated by the thyroid gland (Perlman, Morton, and Chaikoff, 1941). Di-brom trypan blue has been used to localize inflammatory lesions (Moore and Tobin, 1942). This study has been extended (Moore, Tobin and Aub, 1943), by using radioactive di-brom Evans blue as well as di-brom trypan blue in tumor-bearing mice. The uptake of these dyes by the tumor was not selective as judged by the degree of radioactivity.

Antimony 122 and 124. The distribution and metabolic fate of tartar emetic using radioactive antimony has been the subject of several investigations. It has been shown (Bartter, Cowie, Most, Ness and Forbush, 1947) that there was a rapid elimination of antimony during the first two days following a single intravenous injection of tartar emetic. This rapid period was followed by a slow elimination lasting for five days. Eighty percent of the antimony was eliminated by the kidneys and the remainder by the gastrointestinal tract. Later, the same authors studied the blood and tissue distribution of radioactive antimony following injection of tartar emetic. The rat showed a different concentration curve in the blood from that of the dog (Ness, *et al.*, 1947). The rat concentrates the antimony in the liver and later releases it into the blood stream.

Another use of radioactive antimony has been the localization of this element following intravenous injection in dogs infected with *Dirofilaria immitis* (Brady *et al.*, 1945). After a single injection of tartar emetic, examination of various tissues demonstrated the largest amount of radioactive antimony in the liver, the next largest concentration was in the thyroid and the parathyroid while the amount in the filaria was third. It was concluded that these three sites had a specific affinity for antimony.

Iodine 131. One of the chief uses of radioactive iodine in pharmacodynamics has been the study of the site and mode of action of drugs acting on the thyroid gland. The average uptake by the thyroid gland in rats is 56 percent of the administered dose of radioactive iodine in normal rats, 87 percent in thiocyanate-treated animals and only 10 percent in animals given thiouracil (Rawson, Tannheimer and Peacock, 1944). In the same volume of *Endocrinology,* it was reported that thiouracil decreases the capacity of the thyroid gland to concentrate iodine and to convert iodine into thyroxine (Franklin, Lerner and Chaikoff, 1944). The uptake of radioactive iodine has been used for the purpose of assay on the potency of antithyroid drugs in man (Stanley and Astwood, 1947). An interesting series of compounds has been reported, some having more activity than thiouracil, others less. Investigation on thyroid slices has also revealed that thiouracil inhibits the formation of thyroxine from inorganic iodine (Franklin, Chaikoff and Lerner, 1944). Another line of evidence has been supplied which supports the inhibitory action of thiouracil (Couceiro, *et al.*, 1944). It was demonstrated that the thyroids of rats treated with thiouracil did not produce radioautographs following the giving of radioactive iodine. Thiouracil can also suppress hormone production in metastases of adenocarcinoma of the thyroid (Leiter, Seidlin, Marinelli and Baumann, 1946). The only conclusion to this work that can be drawn is that thiouracil acts by preventing the metabolism of iodine by the thyroid gland.

Other uses of Iodine 131 in pharmacodynamics have been to label chiniofon with radioactive iodine and to follow the metabolism of this drug throughout the body (Albright, Tabern and Gordon, 1947). This drug, used in the treatment of amebiasis, is absorbed rapidly and is partially metabolized in the body. During a 48 hour period, 58.6 percent of the ingested drug is liberated intact, while the remainder appears as free iodine and an organic residue. Insulin has been labeled with radioactive iodine and the absorption of this therapeutic agent followed by tracer techniques (Root, Irvine, Evans, Reiner and Carpenter, 1944). It was shown that normal controls and patients with diabetes absorb insulin at an equal rate, while patients with insulin resistance have a delay in absorption. Therefore resistance to insulin is resident in the tissues at the site of injection. Another interesting use of radioactive iodine

has been to show the availability of iodine in dithymol diiodide. This substance, although water-insoluble, can be metabolized in the body and its iodine made available for the thyroid (Baldwin, Thiessen and McInroy, 1947).

A list of organic radioiodo compounds has been published which may be useful for pharmaceutical and cancer research (Bloch and Ray, 1946). Details of preparation are given for radioactive dyes and dye intermediates.

Mercury 197. One application of the use of this isotope has been to measure the concentration of mercury vapor in air (Goodman, Irvine and Horan, 1943). These authors developed a method whereby 0.01 mgm. Hg per cubic meter of air could be detected. The analytical method was sensitive to 10^{-8} grams of mercury.

Gold 198. The distribution of radioactive gold 198 has been studied in human subjects following administration (Sheppard, Wells, Hahn and Goodell, 1947). A high concentration was found in the liver and spleen, an intermediate concentration in the kidney and a low concentration in other organs.

Lead 210. Lead tetraethyl was prepared containing radioactive lead (radium D). Rats were exposed to this compound in inhalation chambers and the total amount of radioactive lead was determined in the entire animal (Mortensen, 1942). It was found that the amount absorbed was proportional to the concentration in the vapor, while the quantity absorbed at a given concentration was proportional to the duration of exposure.

Summary

Numerous examples of the use of tagged elements in pharmacodynamics have been given in this review. It is obvious that pharmacologists have a very valuable tool in isotopes for the explanation of drug action and for the tracing of drugs through the animal body. It is for the latter purpose that pharmacologists will find the greatest use of tagged atoms. Generally, the dosage of a drug is so small that its distribution cannot be determined chemically in the animal body. However, with a tagged molecule, the course and fate of the drug may be followed. Therefore, there can be no doubt that much more use of tagged atoms will be made in pharmacodynamics in the near future.

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CYTOGENETICAL EFFECTS OF INTERNAL RADIATIONS FROM RADIOISOTOPES

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One of the most significant effects of ionizing radiations, such as X- and gamma rays, applied externally to organisms is the production of genetic changes—gene mutations and chromosomal rearrangements. Since the radiations from most radioactive isotopes are fundamentally similar to those utilized as external radiation sources, it is of interest to determine the effectiveness of such radiations in producing genetic changes when these isotopes are present within cells and tissues.

A major problem connected with the utilization of radioisotopes as internal radiation sources is the determination of the radiation dosage. Under certain circumstances this value may be calculated, at least approximately (Marinelli, Quimby, and Hine, 1948). In other cases, attempts must be made to measure the dosage directly. A further problem involves the comparison of the effects of a dosage originating internally with a similar dosage delivered from the outside. Related to these two problems is the possibility that effects of special genetic significance may result from the disintegration of a radioactive atom incorporated in a chromosome. Physical data indicate that the energy of the recoiling atom will be sufficient to cause chemical bond rupture in nearly all cases (Libby, 1947). Thus the position of a disintegrating radioactive isotope within a cell (whether normally incorporated in molecules making up the chromosomes or not) may be of considerable importance.

Investigations of the cytogenetical effects of internal radiations are also of significance in relation to the utilization of radioisotopes in tracer experiments and in therapy. In tracer experiments it is necessary to assume that the radiations do not produce changes sufficient to modify the normal physiology of the system under investigation. It is known, however, that radiations may produce genetic changes which result in profound modifications of normal biochemical processes (Beadle and Tatum, 1941; Beadle, 1945). Thus it is of some consequence to determine the level of isotope activity at which genetic changes become appreciably common. The consideration of such radiation-genetic effects would appear to be particularly relevant in tracer experiments using haploid microorganisms where pronounced changes in the composition of populations are quite possible.

The increasing utilization of radiations from radioisotopes in therapy makes it necessary to consider the cytogenetical effects, particularly those resulting in cellular death, which these radiations may produce, since in certain treatments such effects may well be of primary importance. Further, in all cases of treatment it would seem desirable to evaluate the possible significance of secondary effects such as the induction of inherited genic and chromosomal changes.

MATERIALS AND METHODS

In the experiments to be described, the effects of internal radiations from radioisotopes in producing chromosomal rearrangements have been investigated in the spiderwort, Tradescantia. The pioneer researches of Sax (1938) established the spiderwort as an exceptionally valuable experimental organism for the investigation of radiation-induced chromosomal rearrangements. The recent volume by Lea (1947) summarizes the considerable body of information now available dealing with these effects. In general, the method utilized in past experiments consists of exposing inflorescences to an appropriate external radiation source, making smear preparations of the anthers at definite time intervals following treatment, and analyzing the chromosomal rearrangements visible at the first post-meiotic mitosis in the developing haploid microspores. It is found that the radiations produce breaks in the chromosomes, and the resulting broken ends may either fail to rejoin or may rejoin in various ways to produce visibly aberrant configurations. Two general categories of aberrations are observed: (1) chromatid types, which are produced when chromosomes are broken after they have become effectively double; these are detected from a few hours to approximately 40 hours following treatment. (2) Chromosome types, which result when chromosomes are broken while they are still effectively single; these are observed from about 30 hours to several days after irradiation. It is possible to distinguish several different configuration types in the two categories, depending on the number of breaks involved and the manner in which the broken ends rejoin. These various types have been figured and discussed by Sax (1940) and Catcheside (1945). It was first clearly shown by Sax (1940) in X-ray experiments that both chromatid and chromosome break types could in general be divided into two groups on the basis of their relation to radiation dose. Those exhibiting a linear relation with increasing dose are termed one-hit types and are considered to be produced by single ionizing particles (secondary electrons) while those which increase as the square of the dose are termed two-hit types and result from breaks induced by two separate ionizing particles. Subsequent investigations (Sax, 1941) have shown that a time factor is in-

volved in the production of two-hit types such that at low radiation intensities these types approach a linear relationship to dose.

In the present experiments, inflorescences were removed from Tradescantia plants (clone #5 of Sax was used in all except the first experiment), placed in a solution of a radioactive isotope, and cytological examinations made at intervals following the initiation of treatment in the same manner as in experiments utilizing external radiations. In all experiments inflorescences were shielded from radiation frequency increased so rapidly with continued treatment that within approximately 48 hours an accurate analysis became impossible. It is thus clear that the absorption of radiophosphorus by a plant such as Tradescantia results in the production of extensive chromosomal changes which may be detected in appropriate dividing cells.

In order to obtain data on the kinds and frequencies of different aberration types at various intervals after the initiation of P^{32} uptake, a series of observations was made utilizing the solutions of

TABLE 1. FREQUENCIES OF VARIOUS CHROMOSOMAL REARRANGEMENTS PRODUCED IN TRADESCANTIA MICROSPORE NUCLEI FOLLOWING UPTAKE OF RADIOPHOSPHORUS BY INFLORESCENCES

Time After Start of Experiment	No. of Cells Examined	Chromatid Types per 100 Cells			Chromosome Types per 100 Cells		Total Aber. per 100 Cells
		Cd.	Iso.	Exch.	Dels.	Exch.	
Series B—Initial Concentration of P^{32}: 10 μc./ml.							
24 hrs.	165	1.2	0.0	0.0	0.0	0.0	1.2
48 hrs.	127	5.5	7.9	2.4	0.0	0.0	15.8
74 hrs.	130	9.2	6.2	4.6	0.0	0.0	20.0
4 days	109	29.3	3.7	4.6	2.8	1.0	41.4
5 days	114	19.3	8.8	5.3	1.8	1.0	36.2
6 days	108	25.0	7.4	6.5	0.0	1.0	39.9
8 days	86	26.7	16.3	12.8	5.9	3.5	65.2
9 days	84	23.8	13.0	10.8	1.2	3.6	52.4
Series C—Initial Concentration of P^{32}: 1 μc./ml.							
74 hrs.	146	2.7	0.0	0.0	0.0	0.0	2.7
4 days	136	5.1	0.7	0.7	0.0	0.0	6.5
9 days	116	10.3	0.9	1.7	1.7	1.7	16.3
Series D—Initial Concentration of P^{32}: 0.1 μc./ml.							
4 days	142	2.1	0.7	0.0	0.0	0.0	2.8
9 days	134	1.5	0.8	0.0	0.0	0.0	2.3
Control—no P^{32}: added.							
24 hrs.	644	0.0	0.15	0.0	0.15	0.0	0.30
9 days	626	0.0	0.16	0.0	0.0	0.0	0.16

tions originating from the solution to insure that any effect detected could be ascribed to the actual absorption of the radioactive material.

INITIAL EXPERIMENTS WITH PHOSPHORUS-32

In the first experiments (Giles, 1947) solutions of phosphorus-32 (as $Na_2HP^*O_4$) were used. The P^{32} was added to a nutrient solution containing approximately 0.2 mg./ml. of KH_2PO_4. Solutions of several different initial activities were used, ranging from 100 μc./ml. to 0.1 μc./ml. At the highest activity (100 μc./ml. in the initial solution), examination at the microspore division indicated that within less than 24 hours an appreciable number of chromatid rearrangement types was present. The aberrations lower initial activities. The results of these analyses are summarized in Table 1. The aberration types detected are the same as those produced by external radiation. The following categories and abbreviations have been used in this and subsequent tables: for chromatid types, Cd. (chromatid break), Iso. (isochromatid break), Exch. (all exchange types); for chromosome types, Dels. (terminal and small interstitial deletions), Exch. (exchanges—rings and dicentrics). Aberrations were detected in inflorescences from each of the three radioactive phosphorus solutions tested. Even with initial activities as low as 0.1 μc./ml. of original solution, the aberration frequencies are considerably higher than in the control series. The observed value in the controls agrees

well with that obtained in a previous study using comparable but different non-hybrid plants (Giles, 1940). In the phosphorus experiment, it is clear that the aberration frequency increases with time after start of the treatment. Further, at comparable times after the initiation of treatment the aberration frequencies of inflorescences from the three different solutions are clearly correlated with the original activities of the solutions.

In general the aberration types appear in the same sequence as in X-ray experiments. Chromatid types are observed initially with chromosome types appearing between the third and fourth day. In contrast to the usual radiation experiments with Tradescantia, however, chromatid types do not disappear to be replaced by chromosome types exclusively. Rather they remain the most common type throughout the course of the experiments. This results from the fact that the microspores are being subjected to continuous radiation during their entire development such that chromatid aberrations will be produced when the cell nuclei pass through prophase immediately before the chromosomes are analysed for aberrations at metaphase. The later appearance of chromosome break types, as contrasted with experiments with external radiations when they appear by the second day following treatment, is probably to be explained as a result of a rather slow initial uptake of radiophosphorus, plus the fact that these types exhibit considerably less radiation sensitivity than do chromatid break types (Sax, 1940).

One striking feature of the comparison of chromatid aberration types is that the ratio of exchanges to simple break types (chromatid and isochromatid) is considerably higher on the average than was anticipated from previous investigations utilizing X-radiation. It is not yet possible to state whether this relatively higher yield of exchanges is to be considered a characteristic feature of treatment with internal β radiation. It may be that many of the exchange types are the result of single hits (single β ionization paths). Further, the restitution time of broken ends may not be as short as was originally suggested by X-ray experiments at high intensities (Catcheside, Lea and Thoday, 1946).

The majority of the aberration types studied in these experiments are cell-lethal and result in pollen abortion. It is very probable that viable types similar to the eucentric reciprocal translocations produced in somatic cells in various species of wheat by radiophosphorus absorbed by germinating seeds (Arnason, Cumming and Spinks, 1948) also occur, but no attempt has yet been made to detect these by an examination of meiotic behavior in Tradescantia.

TABLE 2. FREQUENCIES OF VARIOUS CHROMOSOMAL REARRANGEMENTS PRODUCED IN TRADESCANTIA MICROSPORE NUCLEI FOLLOWING UPTAKE OF CARBON-14 BY INFLORESCENCES (FROM SOLUTIONS OF $(NH_4)_2C^*O_3$)

Time After Start of Experiment	No. of Cells Examined	Chromatid Types per 100 Cells			Chromosome Types per 100 Cells		Total Aber per 100 Cells
		Cd.	Iso.	Exch.	Del.	Exch.	
Series A—Initial Concentration of C^{14}: 8.2 μc./ml.							
4 days	59	32.2	23.7	13.5	0.0	1.7	71.1
6 days	25	80.0	68.0	52.0	0.0	0.0	200.0†
Series B—Initial Concentration of C^{14}: 4.3 μc./ml.							
4 days	100	20.0	8.0	7.0	0.0	2.0	37.0
6 days	50	80.0	46.0	26 0	0.0	4.0	156.0
7 days	50	50.0	36.0	38.0	2.0	4.0	130.0
8 days	50	34.0	32.0	26.0	10.0	12.0	114.0
Series C—Initial Concentration of C^{14}: 0.9 μc./ml.							
4 days	86	1.2	1.2	4.6	0.0	0.0	7.0
6 days	85	10.6	2.4	1.2	0.0	0.0	14.2
7 days	80	3.8	0.0	1.3	1.3	1.3	7.7
8 days	150	7.3	3.3	0.7	2.0	3.3	16.6

† Aberration frequency too high to permit very accurate analysis.

EXPERIMENTS WITH CARBON-14

Similar experiments were performed using C^{14} as a radiation source. The C^{14}, originally obtained as BaC^*O_3, was converted into $(NH_4)_2C^*O_3$. The resulting solution was adjusted to pH 7.0 and three solutions of differing activities prepared. The chemical manipulations and solution activity measure-

ments were kindly carried out by Dr. David Anthony. Tradescantia inflorescences were placed in the solutions, each of which was kept under a separate bell jar on the roof of the laboratory building during the course of the experiment. The results of the subsequent cytological analyses are presented in Table 2. Initial observations were not made until the fourth day. As in the phosphorus experiments, aberration frequencies at comparable times are correlated with initial activities, and for each activity there is, in general, an increase in aberration frequency with time. Further, the same general relationships of aberration types are observed.

Correlation of Chromosomal Rearrangements with Radiophosphorus Content

The preceding experiments demonstrate the effectiveness of absorbed radioisotopes in producing chromosomal changes. Further, they indicate that there is a general positive correlation between aberration frequency and initial activity per ml. of a radioactive solution in which inflorescences have been placed.

The next problem is to determine how much radioactive material has been taken up by the plant and to relate the resulting radioactivity to the cytogenetical effect. Such information is essential if quantitative comparisons are to be made between equivalent amounts of external and internal radiation. Consequently, an attempt has been made to develop methods suitable for the simultaneous evaluation of the cytological effects and the "dosage" of radioactivity in a given Tradescantia bud.

The term "dosage" is loosely employed in this paper to express the total number of disintegrations having taken place within a bud and not to denote roentgen equivalents. Before dosage values can be expressed in more conventional terms, it is essential to have a more complete understanding of the effect of the geometry of the bud in terms of the ionizations produced by β particles arising from within the bud.

The most suitable experimental conditions that may be attained will depend on a number of factors. In order to quantitate the radioactivity in a single bud in terms of absolute disintegrations it is essential to obtain conditions of minimum back and forward scattering and of minimum self-absorption of the radiations. These optimum conditions may be realized only by strict observance of the principles involved (Zumwalt, 1947). The samples must be mounted on thin films such as polystyrene in order to obtain minimum back scattering conditions. The buds must be digested so as to remove the bulk of the matter that would contribute to forward scattering and to self-absorption. The observance of these conditions makes it impossible to evaluate the cytological effect on the same bud used for the estimation of the radioactive content unless one resorts to aliquot sampling.

Distribution of P^{32} in Tradescantia Buds

Preliminary experiments were made to determine the distribution of P^{32} among the buds of a single inflorescence as well as among the anthers of a single bud. Inflorescences were placed in a solution containing 10μc./ml. of P^{32}. After a period of four days buds of approximately the same size and shape were selected from single inflorescences, digested with concentrated nitric acid in the presence of 5 mg. of $Na_2HPO_4 \cdot 7H_2O$ and counted in a standard Geiger-Mueller counter. The presence of inactive phosphate is essential to minimize losses of P^{32} by adsorption on the glass surfaces of the equipment. The results indicated that even comparable buds selected

Table 3. Comparison of P^{32} Content of Tradescantia Half-anthers. Buds Removed from Inflorescences after Four Days in P^{32} Solution (10 μc./ml. Initial Activity)

Bud	Half-anther Set	Counts min^{-1}	Average	Percentage Difference from Average
A	1	8,535		
	2	8,769	8,652	1.3
B	1	8,473		
	2	8,228	8,350	1.4
C	1	9,195		
	2	8,555	8,875	3.6
1	a	6,707		
	b	5,959	6,333	5.9
2	a	8,601		
	b	8,946	8,773	2.0
3	a	17,997		
	b	18,728	18,362	2.0
4	a	3,878		
	b	2,979	3,428	13.1
5	a	2,686		
	b	3,332	3,009	10.8
6	a	10,230		
	b	11,183	10,706	4.5
7	a	11,976		
	b	11,776	11,876	0.8
8	a	18,851		
	b	18,758	18,804	0.2
9	a	17,517		
	b	17,389	17,453	0.4
10	a	8,504		
	b	8,529	8,516	0.1

Average % difference = 3.5

TABLE 4. COMPARISON OF P^{32} INDUCED CHROMOSOMAL REARRANGEMENTS IN SINGLE TRADESCANTIA BUDS USING THE HALF-ANTHER METHOD. 10 μc./ml. OF P^{32} INITIAL SOLUTION

Time After Start of Treatment (days)	Bud No.	No. Cells Examined	Chromatid Types per 100 Cells			Chromosome Types per 100 Cells		Total Aber. per 100 Cells	Average Aber. per 100 Cells	% diff. from av.
			Cd.	Iso.	Exch.	Del.	Exch.			
1	A6a	50	0.0	0.0	0.0	0.0	0.0	0.0	2.0	(100)
	A6b	50	2.0	0.0	0.0	0.0	0.0	4.0		
	A9a	50	0.0	0.0	0.0	0.0	0.0	0.0	2.0	(100)
	A9b	50	0.0	2.0	0.0	0.0	0.0	4.0		
2	12a	50	24.0	16.0	30.0	0.0	0.0	70.0	61.3	14.2
	12b	57	15.8	26.3	10.5	0.0	0.0	52.6		
	13a	50	8.0	12.0	2.0	0.0	0.0	22.0	21 0	5.0
	13b	50	2.0	12.0	6.0	0.0	0.0	20.0		
3	15a	50	10.0	18.0	2.0	0.0	0.0	30.0	31.0	3.2
	15b	50	14.0	10.0	8.0	0.0	0.0	32.0		
	16a	50	18.0	42.0	28.0	0.0	0.0	88.0	87 0	1.1
	16b	50	16.0	44.0	26.0	0.0	0.0	86.0		
	17a	50	14.0	28.0	16.0	2.0	0.0	60.0	57.0	5.3
	17b	50	16.0	22.0	16.0	0.0	0.0	54.0		
4	5a	36	44.4	83.3	44.4	2.7	2.7	177.5	180.8	1.8
	5b	25	72.0	64.0	48.0	0.0	0.0	184.0		
	19a	18	5.6	72.2	22.2	0.0	5.6	105.6	113.9	7.3
	19b	18	27.7	55.6	27.7	11.1	0.0	122.2		
6	A5a	50	18.0	32.0	8.0	0.0	6.0	64.0	58.0	10.3
	A5b	50	8.0	20.0	20.0	2.0	2.0	52.0		
	7a	12	75.0	75.0	175.0	0.0	0.0	325.0†	330.3	1.6
	7b	14	71.4	121.4	135.7	0.0	7.1	335 6†		

Average % difference = 5.5

† Aberration frequencies too high to permit very accurate scoring.

from the same inflorescence may vary from the average by as much as 64 percent, and that the values cannot be correlated to the weight of the buds. Such a variation is much too great to permit any correlations to be made and requires that both the P^{32} content and the chromosome aberrations be determined on the same bud.

The next comparison involved a determination of the variation in P^{32} content of the six single anthers from the same bud. Each anther was treated as described previously for the individual buds. The results, although better than those obtained on whole buds, were still not precise enough to permit utilizing three of the anthers for P^{32} analyses and the other three for cytological analysis. The average of the counts per minute for the six anthers was 7,650 ± 8 percent with an extreme range of 24 percent. This variation may be considerably reduced by the method discussed below.

VARIATION IN THE P^{32} CONTENT OF HALF-ANTHERS

In an attempt to obtain more uniform samples suitable both for P^{32} and cytological analysis, it was decided to divide each of the six anthers into halves and to compare the two sets of half-anthers. The division was made under a dissecting microscope with a sharp knife. Fairly good divisions could be made by this method although a small amount of fluid was lost on the knife blade. The amount of P^{32} lost amounted to 1 percent or less. The half-anthers were prepared for counting as before. Table 3 presents the data obtained by this method. When all samples are considered, the variation about the average is of the order of 5 percent. Three out of thirteen buds had a greater error, two of which were above 10 percent. Eight out of thirteen buds had a variation of 2 percent or less from the average. This

TABLE 4—*Continued*

COMPARISON OF P^{32} INDUCED CHROMOSOMAL REARRANGEMENTS IN SINGLE TRADESCANTIA BUDS USING THE HALF-ANTHER METHOD. 10 μc./ml. OF P^{32} INITIAL SOLUTION

Bud No.	Total Breaks per 100 Cells	Average Breaks per 100 Cells	% Difference from Average	General Average: Aberrations per 100 Cells
A6a	0.0	2.0	(100)	
A6b	4.0			2.0
A9a	0.0	2.0	(100)	
A9b	4.0			
12a	100.0	81.6	22.7	
12b	63.1			41.1
13a	24.0	25.0	4.0	
13b	26.0			
15a	32.0	36.0	11.1	
15b	40.0			
16a	112.0	114.0	1.8	58.3
16b	116.0			
17a	70.0	73.0	4.1	
17b	76.0			
5a	225.0	228.5	1.5	
5b	232.0			147.4
19a	133.0	141.5	6.0	
19b	150.0			
A5a	78.0	76.0	2.6	
A5b	74.0			
7a	500.0	489.3	2.2	194.2
7b	478.6			

Average % difference = 6.2

value falls within the expected error of the instrument.

RELIABILITY OF THE HALF-ANTHER METHOD FOR CYTOLOGICAL ANALYSES

Utilizing the half-anther method just described, it is quite feasible to use one group of half-anthers for cytological observations by the usual smear techniques. Thus the initial cytological problem is concerned with determining the reliability of cytological analyses by this method as was done for the P^{32} determination. The results of such analyses are shown in Table 4. All slides were scored under code and the data compilations made after all scorings were completed. Comparisons have been made on the basis of both aberrations (of all types) per 100 cells and breaks per 100 cells. Calculations of breaks per 100 cells have been made in accordance with the results of experiments utilizing X-radiation of relatively high intensity which indicate that chromatid exchanges and chromosome dicentrics and rings are principally two-break (two-hit) types, whereas chromatid and isochromatid types, as well as the majority of terminal and interstitial chromosome deletions, are one-hit types. In the present experiments, however, the radiation intensity has been considerably less than in such X-ray experiments and many of the two-break types are undoubtedly the result of single hits (single ionization paths). Thus it is probable that the comparison of aberrations per 100 cells is the more valid measure of variability. For both comparisons, when all samples are considered the variations about the average is of the order of 5 to 6 percent. These comparisons indicate that the precision of the cytological results is about the same as that of the phosphorus determination by this method.

Certain general observations regarding the types and frequencies of various aberration types may also be made on the basis of the data in Table 4. Chromatid aberration types are the only ones recorded in the observations at two days and three

days (with one exception) after the initiation of treatment. Chromosome types first appear regularly on the fourth day. These results thus agree with those obtained in previous similar experiments with P^{32}. The average frequency of aberration types per 100 cells increases with increasing time of exposure to the P^{32} solutions as the figures in the last column of Table 4 indicate.

The aberration frequencies recorded with these experiments are in general higher than those obtained in previous experiments in which solutions of frequency of chromosomal aberrations. This is particularly true within a single experimental series, and especially at higher activity levels where the statistical reliability of both measurements is better. The aberration frequency in the control utilized in series PV is considerably higher than that obtained in previous controls, but is based on the analysis of a much smaller number of cells.

It is to be noted that the length of treatment varies, and consequently the cytological effect cannot be directly related to the activity as recorded

TABLE 5. COMPARISON OF ACTIVITY MEASUREMENTS (P^{32}) AND CHROMOSOME ABERRATIONS IN SINGLE TRADESCANTIA BUDS USING THE HALF-ANTHER METHOD

Series No.	Bud No.	Orig. Act. of P^{32} Solution	Days after Start of Treatment	No. Cells Examined	Chromatid Types			Chromosome Types		Aberr. per 100 Cells	Activity per Bud c./m.
					Cd.	Iso.	Exch.	Dels.	Exch.		
PIV	B-12	1.0 μc./ml.	8½	100	14	11	3	2	1	31.0	10,184
	B-15a	1.0 μc./ml.	8½	146	6	4	0	0	1	7.6	5,707
	B-15b	1.0 μc./ml.	8½	113	1	2	0	0	0	2.7	3,489
	D-20	0.01 μc./ml.	8½	169	0	0	0	0	0	0.0	43
PV	A-2	0.1 μc./ml.	8	250	0	0	0	1	0	0.4	684
	A-6	0.1 μc./ml.	8	124	4	1	0	0	1	4.8	1,144
	A-10	0.1 μc./ml.	8	164	1	1	0	0	1	1.8	336
	Control	0.0 μc /ml.	8	185	0	1	0	0	0	0.5	17
PVI	9	10.0 μc./ml.	2	100	21	17	11	0	0	49.0	40,390
	10	10.0 μc./ml.	2	100	16	11	3	0	1	31.0	19,707
	5	10.0 μc./ml.	3	12	9	5	17	0	0	257.3†	96,000
	3	10.0 μc./ml.	3	100	16	21	6	0	0	43.0	17,130

† Aberration frequency too high for very accurate analysis.

about the same initial activity were utilized. The principal reason for such differences is probably the fact that the present solutions were essentially carrier-free, whereas an appreciable amount of inert phosphorus was added to the original solutions. Further, the initial experiments were carried out with a separate clone of plants and under different external environmental conditions, which must necessarily influence the uptake of P^{32}.

COMBINED P^{32} AND CYTOLOGICAL ANALYSES BY THE HALF-ANTHER METHOD

The previous discussion indicates that the half-anther method should be sufficiently accurate to permit correlations to be made between P^{32} content and chromosome breakage. Consequently, it is of interest to examine the results obtained when both activity measurements and cytological analyses have been obtained from single buds in this manner. Such data are presented in Table 5. The activity measurements are given for whole buds, the recorded count value for a set of half-anthers being doubled and added to that for the remainder of the bud. It is evident that there is a definite positive correlation, between the activity of a given bud and the at any one time. Rather it will depend on the total number of disintegrations that have taken place within the bud. This in turn will depend on the rate of uptake of P^{32}, which must be determined for each bud.

RATE OF P^{32} UPTAKE IN TRADESCANTIA BUDS

In order to obtain the P^{32} uptake curve the standard mounting of the Geiger-Mueller tube was slightly modified so that the radiations from the bud

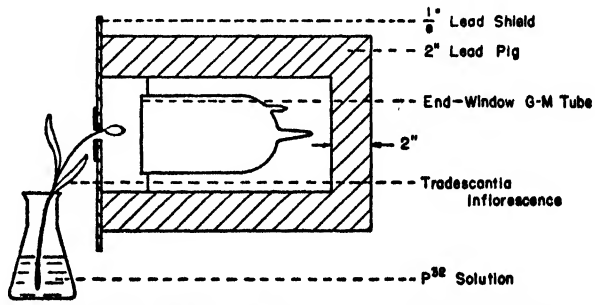

FIG. 1. Geiger-Mueller tube mounting for recording the rate of P^{32} uptake in a Tradescantia bud.

could be measured while the radiations from the rest of the plant and the solution could be shielded (Fig. 1). A standard 1024 scaler connected to an Esterline Angus recorder was employed to give a relative time-activity curve. Two such curves are shown in Figure 2. To the P^{32} solution used for curve 2 was added about 10 mg. of $Na_2HPO_4 \cdot 7H_2O$ per ml. The addition of inactive phosphorus to the solution markedly retarded the rate of P^{32} uptake as could be expected from the principle of isotopic dilutions. The other solution, although not carrier-free, contained very little inactive phosphorus.

In order to obtain an absolute count in terms of disintegrations the bud is then dissected. Six half-anthers are employed for the cytogenetic count and the remaining six half-anthers and other parts of the bud may be digested separately with concentrated nitric acid in the presence of phosphate for the determination of the total number of disintegrations for the whole bud at the end of the run. This value may then be employed to correct the relative area under the time curve to absolute number of disintegrations that have taken place from the start to the end of the experiment. This final value then becomes related to the true dose of radiation the bud has received.

Values obtained by the method will have a certain degree of uncertainty depending on the experimental conditions and on the validity of certain assumptions associated with such measurements. These assumption are that the P^{32} will be equally distributed among the various parts of the bud during the uptake period, the background value will remain constant throughout the run, and the radiations backscattered from the shield and originating in the unshielded portions of the stem will produce a negligible biological effect. At present we have not completely tested the validity of these assumptions. Preliminary data indicate that they are in part not fully justified and that some further work is required both in the design of the instrument and in the determination of the degree of uncertainty present in such determinations. Nevertheless it is apparent that the results obtained in this way will be much more representative than those correlating the cytogenetic changes with the activity of the solution in which the inflorescences are placed.

There is one further difficulty with the method. The microspore nuclei in the bud may not be in the proper dividing state when the counting is terminated. This point can be determined only by cytological examination. The bud must be left in position in the instrument and can be examined only superficially from time to time during phosphorus uptake provided care is taken that its geometrical position and shape with respect to the tube are not altered. Hence, it may be expected that a fair number of buds will not be usable for cytogenetic

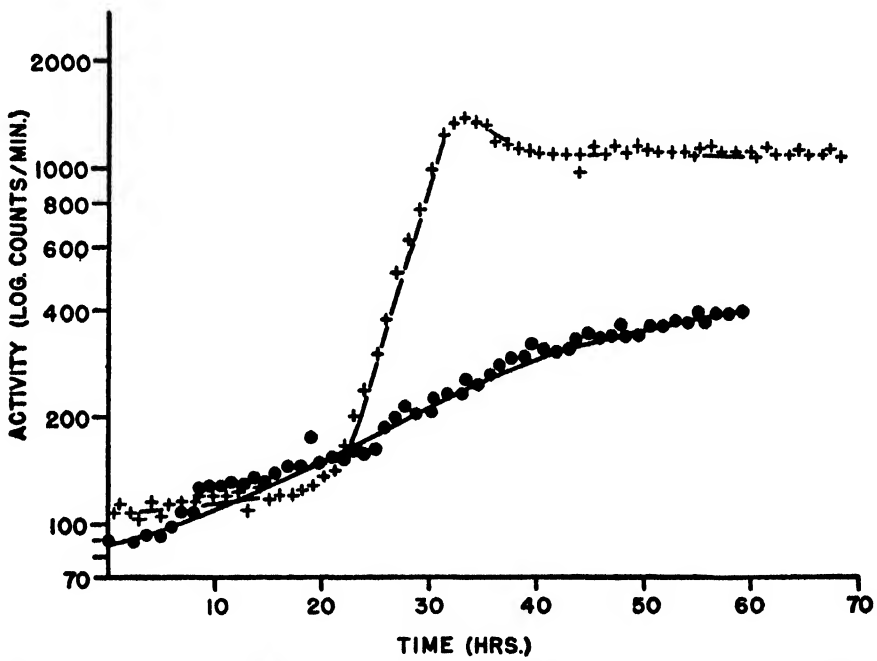

FIG. 2. Uptake curves of P^{32} in Tradescantia buds. Curve 1 (+++)—solution practically carrier free; Curve 2 (···) —10 mg. of $Na_2HPO_4 \cdot 7H_2O$ added per ml. of solution.

analysis. This difficulty can be largely eliminated by the use of a multiple-channel count rate recorder. Such an instrument will permit the simultaneous analysis of P^{32} uptake in several buds, of which those in the appropriate state of mitosis can be used for cytological analysis.

Data correlating chromosome aberrations with the total number of disintegrations taking place within a single bud from the time an inflorescence is immersed in the P^{32} solution to the time the bud is sampled are not yet available. However, it is clear from the preceding tables that there is a distinct trend showing an increase in the number of aberrations occurring per 100 cells with time. It is apparent that much of the variation found among buds for any given treatment would have been considerably decreased had the data been correlated with the total number of disintegrations that had taken place.

Summary

Studies have been made of the effects of radiations from radioisotopes taken up in solution by inflorescences of the spiderwort, Tradescantia, in producing chromosomal rearrangements in microspore nuclei. Beta radiations from both phosphorus-32 and carbon-14 are effective in producing chromosomal changes which are cytologically indistinguishable from those produced by external radiation sources such as X-rays. An increase in aberration frequency over that in controls has been detected in inflorescences placed in solutions of initial activities from 100 to 0.1 μc./ml. The sequence of aberration types is fundamentally similar to that observed in previous X-ray experiments, but the relative frequency of chromatid exchanges is considerably higher.

The foundations for a method of correlating the frequency of chromosome aberrations in a given bud with the P^{32} activity of the bud have been laid. It is shown that the best method of sampling buds for the simultaneous determination of P^{32} activity and aberration frequency involves the use of two sets of half-anthers. Using this method, the sampling error for both P^{32} determination and chromosome aberations is of the order of 5 percent. The total number of disintegrations taking place within a single bud from the time the inflorescence is placed in a P^{32} solution to the time the bud is removed for chromosomal analysis may be determined by slightly modifying the standard mounting of the Geiger-Mueller tube and utilizing a recording counter.

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Discussion

KAUFMANN: In the preceding discussion it has been reported that low temperature will inhibit lethal effects or physiological disturbances attributable to ionizing radiations. Low temperature, however, may enhance rather than inhibit production of such chromosome aberrations as Dr. Giles has described. Several years ago Sax and Enzmann showed that irradiation of microspores of Tradescantia at low temperature will increase the frequency of aberration as compared with that obtained at high temperature. This result was attributed to a delay in reunion that permits broken ends to form new associations more readily at a low temperature than at a higher one. A similar effect of low temperature during irradiation in increasing the frequency of chromosomal rearrangement has been obtained by several workers in experiments involving treatment of chromosomes of the mature spermatozoa of Drosophila.

On the other hand, exposure of chromosomes to near infrared radiation before exposure to X-rays will also increase the frequency of rearrangement. Recently Fabergé reported that change in temperature in either direction, immediately preceding X-ray treatment, will increase the yield of aberrations in Tradescantia. It is thus apparent that a variety of factors, including low and high temperature, may influence the process of induced structural rearrangement so as to produce comparable end results. Apparently the supplementary treatment operates to influence the quality of the induced breaks and the subsequent behavior of the breakage ends.

STUDIES ON THE MECHANISM OF PROTEIN SYNTHESIS WITH RADIOACTIVE CARBON-LABELED COMPOUNDS[1]

DAVID M. GREENBERG, FELIX FRIEDBERG,[2] MARTIN P. SCHULMAN AND THEODORE WINNICK

INTRODUCTION

The outstanding importance of the proteins in the structure of protoplasm, biocatalysis, reactions of immunity, and the control of heredity makes the quest for the understanding of the reactions required for the synthesis and the control of the synthesis of proteins of the greatest interest to biochemists.

The mechanisms concerned with the synthesis of proteins also, obviously, are intimately associated with the processes of growth and regeneration. Indeed, the accumulating evidence that all the tissues of the body are in a state of constant flux makes it clear that even the maintenance of adult organisms involves a continuous process of protein synthesis and a balance between the reactions of tissue anabolism and catabolism.

It may be safely surmised that the primary reactions of protein synthesis are enzymatic in nature. These must be universal in their distribution in nature and probably are identical, or at least very closely related, in all organisms, be they microorganisms, plants, or animals. The development of enzymatic reactions for protein synthesis may be presumed to have coexisted with the genesis of the most primitive organisms.

The subject of protein synthesis has been reviewed recently in a number of publications (Northrop, 1946; Northrop, Kunitz and Herriott, 1948). Among the more important hypotheses that have been advanced to explain the mechanism of protein synthesis are the following:

1. It has been suggested that there is a catalytic reversal of the conditions for the hydrolysis of the peptide bond under the influence of proteolytic enzymes. No external source of energy would be required for this mechanism. The synthetic reaction would be favored by a high concentration of the reactants (amino acids) and removal of the products of synthesis (protein, peptides) from the sphere of reaction as soon as formed.

2. The second type of reaction mechanism takes into account the necessity of providing an external source of energy to drive the reaction in favor of synthesis. Thus, it has been proposed that amino acids are first phosphorylated, producing compounds with a high energy phosphate bond content that then condense with other amino acids or peptides to build up peptide chains. A second proposal is that acetylated amino acids are formed from pyruvate and these condense with carboxyl groups to yield peptide groups. Still another proposal is that the peptide bond is formed through the condensation of amino acid amides with a keto acid to form a dehydro peptide which is subsequently hydrogenated to yield a peptide. While exact mechanisms are still unknown, it now appears clear that protein synthesis is dependent upon energy yielding reactions supplied by aerobic respiration in animal cells.

It should be noted that the theories of protein synthesis and the experimental work discussed in this paper refer only to the gross mechanism of the formation of peptide bonds between amino acid residues. The mode of production of the finer structure and characteristic specificity of the many proteins that have been biochemically investigated is an even deeper mystery than peptide bond formation.

It is the purpose of this article to present a summary of some investigations relating to the problem of the mechanism of protein synthesis by enzymatic reactions, the role of protein synthesis versus protein degradation in growth and regeneration, and the effect of certain hormones on protein anabolism.

A. THE PROCESS OF AMINO ACID INCORPORATION INTO PROTEIN OF LIVER HOMOGENATES

Incubation of tissue slices in an oxygenated nutrient medium in the presence of radioactive methionine (Melchior and Tarver, 1947), alanine (Frantz, Loftfield and Miller, 1947) or glycine (Winnick, Friedberg and Greenberg, 1947) leads to an uptake of the labeled amino acid into the protein of the slices. Recently it was observed that the C^{14} of labeled glycine was incorporated into the protein of cell-free *homogenates* of liver and other tissues (Winnick, Friedberg and Greenberg, 1948).

Homogenates of a variety of animal organs have been found capable of incorporating the C^{14} of labeled glycine into protein. Table 1 gives illustrative data for the mouse.

Both liver slices[3] and homogenates exhibit certain common properties with respect to the utilization of radioactive glycine. These are:

[1] Aided by grants from the American Cancer Society (recommended by the Committee on Growth), the National Cancer Institute, and the John and Mary R. Markle Foundation.

[2] Present address: Howard University, Washington, D.C.

[3] The comparison between tissue slices and homogenates has been made by Mr. P. Siekevitz of our laboratory.

1. Incubation in an oxygenated Krebs-Hensleit medium of pH 7.4 at 37° leads to a rapid incorporation of C^{14} into protein.

2. The uptake process is drastically inhibited by lack of oxygen, or by the presence of cyanide or azide, and is completely abolished by heating to 100°.

3. Certain ions, calcium in particular, are activators of the system.

4. A part of the labeled glycine in the nutrient system is metabolized to serine, and the latter is incorporated into protein along with the glycine. Approximately one-third of the C^{14} appearing in either slice or homogenate protein represents serine, while the remainder is chiefly due to glycine.

5. Lipid fractions, extracted with alcohol-ether, contain C^{14}. The latter is present in the form of serine bound through its hydroxyl group, presumably as phosphatidyl serine (Winnick, Peterson, and Greenberg, 1949).

Homogenates offer certain advantages over slices for the study of the process of protein synthesis:

1. A greater uniformity of sampling is possible, since aliquots of a homogenate may be pipetted. Slices may vary considerably in thickness, and consequently in activity.

2. Homogenates are suited for the study of the role of various ions and metabolites in the incorporation process, since the factor of cell membrane permeability is eliminated.

3. Perhaps, most important, cell-free homogenates are a step in the direction of obtaining isolated preparations capable of promoting protein synthesis.

One difference in the behavior of liver slices and homogenates is that the latter exhibit a high rate of glycine utilization for only about one hour, and thereafter rapidly lose activity. Slices, on the other hand, possess undiminished activity for several hours. It is noteworthy, however, that homogenates of rat embryos incorporate the C^{14} of glycine at a very rapid and approximately constant rate for a period of at least four hours at 37°.[4]

In experiments with carboxyl-labeled glycine and liver homogenate (Winnick, Peterson, and Greenberg, 1949), the radioactive protein formed in the incubations appeared to represent a true incorporation of amino acid units into protein molecules, as judged by the following criteria:

1. No $C^{14}O_2$ was evolved from the unhydrolyzed protein upon heating with ninhydrin solution at pH 2.5 and 100°. Following complete (acid or alkaline) hydrolysis, virtually all of the C^{14} of the protein was released in the carbon dioxide by ninhydrin.

2. Hydrolysis of the protein to the peptide stage with a crystalline proteinase, such as pepsin or trypsin, and subsequent treatment with ninhydrin liberated no $C^{14}O_2$. Hydrolysis with a combination of proteinases and peptidases, followed by ninhydrin treatment released approximately 75 percent of the C^{14}.

3. The major portion of the C^{14} was accounted for by labeled amino acids (glycine and serine) isolated from the completely hydrolyzed protein with carriers.

4. The removal of nucleic acids, from the protein produced no appreciable decrease in the radioactivity of the latter.

The study of the roles of the macroparticles (nuclei, mitochondria, etc.) and the soluble or nonsedimentable fraction of liver homogenates in the amino acid incorporation process is rendered difficult by the fact that washing with isotonic salt solution reduces the activity of the particles considerably. Unwashed particles, separated from a 1:1 homogenate of liver and Krebs-Hensleit solution by centrifuging at 4000 R.P.M. in an angle head refrigerated centrifuge, had approximately the same activity as the unfractionated homogenate. However, the supernatant (nonsedimented phase) had virtually zero activity. Additional experiments are required before the role of the macroparticles can be evaluated.

The homogenates lose activity on standing. They are sensitive to salt concentration, and are inactivated by freezing or lyophilizing.

The information accumulated to date permits few definite conclusions relative to the mechanism of amino acid incorporation into the proteins of tissue slices or homogenates. The C^{14} of glycine and alanine is incorporated into liver slice protein at different rates, the process being several times more rapid with the glycine C^{14} (Zamecnik and coworkers, 1948). The same situation was observed with liver homogenates. Possible explanations of these differences are: (1) Glycine forms labeled serine, which augments the degree of C^{14} incorporation. (2) Amino acids may exhibit different turnover rates in proteins. (3) Alanine may be present in lower percentage in liver proteins than glycine.

It is of interest to know whether amino acids enter a protein molecule individually, or require the participation of other amino acids. Preliminary experiments indicated that a mixture of the various amino acids (excepting glycine) added to a homogenate containing labeled glycine inhibited the uptake of the latter. However, certain of the added amino acids were racemic compounds, and it is possible that the D-forms were responsible for the inhibitory effect. In subsequent experiments, a mixture of amino acids entirely of the natural (L) configuration, stimulated the uptake of labeled glycine into homogenate protein.

There is considerable interest in the question as to whether amino acid incorporation involves intermediate peptide formation. Experiments with radioactive lysine and leucine (Borsook *et al.*, 1948 a and b) in liver homogenates have shown the formation of labeled peptides. While the accumulation of these

[4] Unpublished data obtained by Mr. E. Peterson of our laboratory.

substances may have been unduly promoted by the amino acid mixture added to the system, it is quite possible that the formation of such peptides is intimately related to protein synthesis.

There is still no definite proof that phosphate bond energy is required for protein synthesis. Adenosine triphosphate did not promote the uptake of labeled glycine in liver homogenates anaerobically. However, the inhibitory effect of dinitrophenol on the incorporation of alanine into liver slice protein has been attributed to an interference with the synthesis of energy-rich phosphate bonds (Frantz, Zamecnik, Reese, and Stephenson, 1948). Some type of oxidative process is certainly suggested by the fact that oxygen is required, and that cyanide and azide are strong inhibitors of amino acid incorporation.

TABLE 1. RELATIVE C^{14} UPTAKE BY PROTEINS OF MOUSE TISSUE HOMOGENATES AFTER INCUBATION WITH LABELED GLYCINE†

Counts per mg. protein per minute				
Brain	Cardiac muscle	Kidney	Liver	Spleen
3.87	1.88	7.56	7.04	49.1

† Organs of 6 mice were pooled and duplicate samples were determined in each test. The flasks were incubated for 90 min. at 37°C. in an O_2 atmosphere; 250 mg. of tissue were homogenized in 4 ml. standard medium and incubated with 0.1 mg. glycine* (70,000 counts per minute per flask).

B. GROWTH AND REGENERATION

Investigation of the fate of isotopically labeled amino acids in recent years makes it evident that the proteins of the body are in a continuous state of flux, constantly undergoing both synthesis and breakdown. In the maintenance of the static organism as a whole, or of its constituent organs and tissues, it is quite obvious that there is a very precise balance between protein synthesis and degradation leading to a dynamic steady state. In the growing animal and in regenerating tissues there is a net increase of protein. This could result either from an increase in the rate of protein synthesis or a retardation of protein breakdown. This is a question that has been recently raised which is of singular significance for the clarification of the problem of growth.

The experimental solution of whether growth is the result of a stimulation of protein synthesis or a retardation of its breakdown is fraught with great difficulties, regardless of whether it is attacked by the newer isotopic tracer methods or by the older standard metabolic procedures. In isotopic experiments on the intact animal, interpretation of turnover rates is complicated by such factors as permeability, blood supply, and the effect of catabolism (after the lapse of any considerable interval of time during which a high concentration of label has been introduced into the product).

The importance of permeability is exemplified by the spuriously low rates of turnover of labeled amino acids by brain when injected into the intact animal because of the blood brain barrier. Examples of low metabolic activity due to a low blood circulation are offered by bone and the skin. When the product has incorporated a high content of the label, the rate of the return of the label to the metabolic pool by degradation reactions assumes prominence and complicates the determination of the rate of synthesis.

It should also be pointed out that where an increased rate of protein synthesis can be demonstrated in a growing tissue, it does not preclude the involvement of a decelerated rate of protein decomposition. The role of protein degradation in growth is most difficult to demonstrate. The isotopic tracer techniques lend themselves far less readily to this end than to the estimation of incorporation.

To secure evidence on the mechanism of growth, a study of the intrinsic synthetic rates of tissues has been undertaken in our laboratory on cell free tissue homogenates. This eliminates such complicating factors as permeability and blood supply. The amino acid used was C^{14}-labeled glycine. From the evidence obtained it appears evident that certain growth processes such as embryonic growth, can only be explained by a high rate of protein synthesis and a tremendous preponderance of synthetic over degradative processes. As evidence may be cited the results contained in a recent publication (Friedberg, Schulman and Greenberg, 1948) in which it was found that the incorporation of C^{14} from labeled glycine into the protein of embryonic rat livers was over a hundred fold as great as that of adult liver. Similar experimental evidence is given in Table 2, on the rate of C^{14} incorporation of chick embryo and embryonic liver and brain.

TABLE 2. INCORPORATION OF C^{14} FROM LABELED GLYCINE INTO THE PROTEIN OF HOMOGENATES OF EMBRYONIC AND NEWLY HATCHED CHICK TISSUE†

Counts per mg of protein per minute				
5 day embryo	13 day embryo		Newly hatched chick	
	Liver	Brain	Liver	Brain
176.2	48.2	102.7	32.8	38.2
182.8	52.5	104.8	28.8	38.4

† Incubation procedure was the same as given in footnote to Table 1.

In slower growth processes, the differences in incorporation are not quite as striking. However, the homogenates and slices of regenerating liver of par-

tially hepatectomized rats were found to have a distinctly higher rate of C^{14} incorporation, and similar results were observed for the S^{35} of labeled methionine in rat liver slices. The data are given in Table 3. These experiments, while they suggest that protein synthesis is increased in the regenerating liver as well as the growing organism, do not preclude the possibility that a decrease in the rate of protein decomposition may also be an important factor.

TABLE 3. INCORPORATION OF C^{14} FROM LABELED GLYCINE INTO THE HOMOGENATES AND OF S^{35} INTO THE TISSUE SLICES OF REGENERATING LIVER†

C^{14} in liver homogenate protein; counts per mg. protein per minute		S^{35} in liver slice protein Specific activity×100 / dose		
Rat No.	Preoperative	Postoperative	Rat No.	Preoperative	Postoperative
1	6.53	7.62	6	0.041	0.073
2	5.21	7.83	7	0.059	0.095
3	7.08	10.50	8	0.059	0.083
4	6.04	8.58	9	0.089	0.088
5	5.58	7.31			

† Partial hepatectomy carried out as described by Crandall and Drabkin (1946). Portions of regenerating liver for tests were removed 3 days after operation. Experiments with homogenates were performed as mentioned in footnote to Table 1. Experiments with S^{35} on liver slices were performed in the same manner as those with C^{14} on liver homogenates.

C. HORMONAL CONTROL OF PROTEIN SYNTHESIS

With increasing complexity of organization in the animal kingdom other mechanisms for the precise control of the interrelationships of metabolism among the various organs and tissues of the body came into being, namely, the endocrine glands. The endocrine system, apparently, had its origin with the vertebrates and reached its full flower among the mammals.

Certain of the hormones of the endocrine system exert an influence on protein metabolism. The growth hormone of the pituitary, the androgens and estrogens, and, in maintenance amounts, the thyroid hormone promote protein anabolism, while the adrenal cortex, and excess of the thyroid hormone, favor protein catabolism.

No definitive evidence for a direct control of any enzyme reaction by a hormone has yet been established. It may be determined in due time that certain of the hormones produce their characteristic actions by direct or indirect activating or inhibiting mechanisms of specific enzyme reactions.

In the field of protein synthesis we wish to suggest that none of the hormones exert their action through a direct effect on the enzymic mechanisms of protein synthesis. In other words, in our estimation, none of the endocrine glands produce what may be specifically designated as a *protein hormone.* It is suggested, instead, that the effect of a hormone is on some particular type of cell or organ, the *target organ* for that hormone. If the hormonal effect induces a hyperplasia or actual growth of the target organ, this is associated with an increased rate of protein synthesis; if the endocrine state is one of dystrophy, protein synthesis is retarded.

Mention of a protein hormone immediately brings to mind the growth hormone of the anterior pituitary as the most likely example. Administration of purified hypophyseal growth hormone to hypophysectomized and normal animals initiates an increase in body weight and a correspondingly greater nitrogen retention. However, the increase in organ and tissue weights are not uniform.

Friedberg and Greenberg (1948) have presented evidence that growth hormone induces an increased protein anabolism in skeletal muscle. The incorporation of S^{35} from labeled methionine into the protein of skeletal muscle six hours after its injection in the animal was increased about 70 percent by administration of growth hormone. On the other hand, liver showed no such marked increase in amino acid uptake under the influence of growth hormone.

TABLE 4. EFFECT OF TESTOSTERONE PROPIONATE ON INCORPORATION OF S^{35} INTO MUSCLE AND COMB OF CAPONS†

	Number	S^{35} Activity Specific activity×100 / Administered dose	
		Muscle	Comb
Orchidectomized chick No treatment	6	2.61±0.37	6.07±0.97
Orchidectomized chick Testosterone treated	6	2.36±0.43	26.2 ±1.87

† Values are arithmetic means ± standard error.

Control and experimental chicks were orchidectomized about 5 weeks after hatching and 1 week before start of injections of testosterone. Doses of 12.5 mg. of testosterone propionate (Schering) were injected subcutaneously on successive days for 6 days. On day of sacrifice, the capons were fasted 12 hrs. and injected with 0.5 ml. saline containing 0.5 mg. of S^{35} labeled methionine (4,410 counts per min.) via the wing vein. They were sacrificed 4 hrs. later. The methods of preparing the tissues, analyzing for sulfur and determining the radioactivity of the isolated sulfur (as $BaSO_4$) are as described by Tarver and Morse (1948). The authors gratefully acknowledge their indebtedness to Dr. H. Tarver for supplying the radioactive methionine, to Dr. C. R. Grau for preparing the capons, and to Dr. W. H. Stoner of the Schering Corporation for furnishing the testosterone propionate.

It should be noted that in the period of six hours in the above experiments, when the S^{35} content of the tissue is increasing sharply and protein catabolism is removing but little of the S^{35}, the specific

activity of the labeled sulfur is essentially a measure of protein synthesis.

A striking illustration of the hypothesis proposed here, that when a target organ is stimulated to growth or hypertrophy there is an accompanying increase in protein synthesis, is offered by the action of the androgen, testosterone. Attempts to demonstrate anabolism of protein by testosterone in experimental animals by the classical metabolic methods have been inconclusive. In experiments with labeled methionine carried out on the capon it is shown that in the skeletal muscle incorporation of S^{35} is uninfluenced whereas in the comb, which is a target organ for testosterone, there is a striking increase in the specific activity of the sulfur of the tissue. This is shown in Table 4.

The authors are of the opinion that the action of the lactogenic hormone of the hypophysis and of the estrogens on nitrogen metabolism can be explained in similar fashion.

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Discussion

Menkin: I was particularly interested in your curve of protein synthesis where you show that the rate of synthesis is greatest in the embryonic stage. This reminds me of Minot's growth curve published some forty years ago or so. Minot showed that the rate of growth is a function of the rate of differentiation with development of the organism. His curve was quite similar to the one you have shown on the rate of protein synthesis. I wonder whether the rate of differentiation with development may not likewise be correlated with the rate of protein synthesis.

I should also like to know whether you have carried out such protein synthesis studies on the growth of tumors.

Greenberg: No correlation has so far been attempted between the activity of incorporation of labeled amino acids and the rate of mitosis. Studies on the rate of protein synthesis of tumors with labeled amino acids are now under way.

THE BIOLOGICAL OXIDATION OF FATTY ACIDS

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It is a striking commentary that, at present, the only clearly identified products arising from the catabolism of fatty acids are the ketone bodies and carbon dioxide and water. Even acetoacetate can no longer be considered a direct intermediate of fat oxidation since Lehninger (1946a) has demonstrated that while washed liver homogenates can completely oxidize fatty acids, acetoacetate is metabolically inert in this same system. Except for our knowledge that products of fatty acid oxidation are converted to carbon dioxide and water by way of the tricarboxylic acid cycle, we have no understanding of the precise chemical nature of any direct intermediate of fatty acid catabolism.

The oxidation of fatty acids is currently believed to involve cleavage of the aliphatic chain into small fragments containing two carbon atoms. These two-carbon units may (1) recondense in the liver to form acetoacetate or (2) become oxidized to carbon dioxide and water via the tricarboxylic acid cycle. The synthesis and accumulation of acetoacetate by the liver serve to differentiate it from extrahepatic tissues which apparently do not form appreciable amounts of acetoacetate. These tissues can, however, oxidize acetoacetate (Stadie, 1945) and fatty acids (Lehninger, 1946b) to carbon dioxide and water whereas, in liver, acetoacetate is relatively inert. It is convenient, therefore, to consider three main aspects of this field: (1) the mechanisms by which fatty acids are converted to two-carbon units which can serve as precursors of acetoacetate, (2) the reactions involved in the complete oxidation of these two-carbon units to carbon dioxide and water and (3) evidence pertaining to the nature of the two-carbon intermediate.

The Formation of Two-Carbon Units and Acetoacetate from Fatty Acids

The classical experiments of Knoop as well as Dakin have long supported the belief that two-carbon fragments may be successively split off from fatty acids. How this is accomplished is still unknown. Numerous investigators (Leloir and Munoz, 1939; Munoz and Leloir, 1943; Lang, *et al.*, 1939; Fontaine, 1943; Quagriarello, 1932, 1941; Mazza and Stolfi, 1933; Mazza and Marfori, 1941) have reported the preparation of crude enzyme systems capable of desaturating fatty acids. Whether α, β unsaturated acids are direct intermediates in this oxidative cleavage has not been established. Mazza and Marfori (1941) have obtained spectroscopic evidence indicating that oxidation occurred in the alpha-beta position. In adipose tissue there likewise appear to be present dehydrogenase systems capable of oxidizing phospholipids (Shapiro and Wertheimer, 1943). It is generally assumed that this first reaction involves dehydrogenation at the alpha-beta position. The subsequent formation of a beta hydroxy-acid is not considered likely in view of the work of Jowett and Quastel (1935a) which eliminated beta hydroxy-butyric acid as an intermediate in the conversion of butyrate to acetoacetate.

That a carbonyl group is directly or indirectly produced in the beta position is certain if one consider only the nature of the resulting metabolic products. Whether or not alternating carbonyl groups are formed throughout the chain length is purely hypothetical at the moment and represents a theoretical mechanism which is no longer necessary in the light of more recent information.

Breusch (1943) has suggested that fatty acids after conversion to β-keto acids are able to condense with oxalacetate and subsequently form citric acid along with a residual fatty acid shorter by two carbon atoms. This postulated enzyme system (citrogenase) appeared to be present in large amounts in muscle, kidney and brain with little present in liver. The relative absence of this enzyme from liver suggests that there is a different enzyme system functioning in the liver and that a different type of cleavage may occur in this important organ.

Preliminary evidence that β,δ-diketo acids may be involved in the oxidation of fatty acids has been obtained by Breusch and Ulusoy (1947) who reported that synthetic β,δ-diketo hexanoic acid is rapidly converted to acetoacetate by liver homogenates. α,γ-diketo hexanoic acid, however, was found to be metabolically inert in this system. An earlier report by Lehninger (1944) demonstrated that α,γ-diketo octanoic acid was similarly inactive when incubated with liver tissue.

Further support for cleavage at the beta position was obtained by Schoenheimer and Rittenberg (1937) who demonstrated the conversion in the living organism of deuterio stearic acid to deuterio palmitic acid. Stetten and Schoenheimer (1940) confirmed this result and also reported the conversion of deuterio stearic to deuterio palmitic, lauric and myristic acids. That fatty acids may yield acetyl groups has been demonstrated by Bloch and Rittenberg (1944). Bloch (1947) has reviewed this subject in great detail.

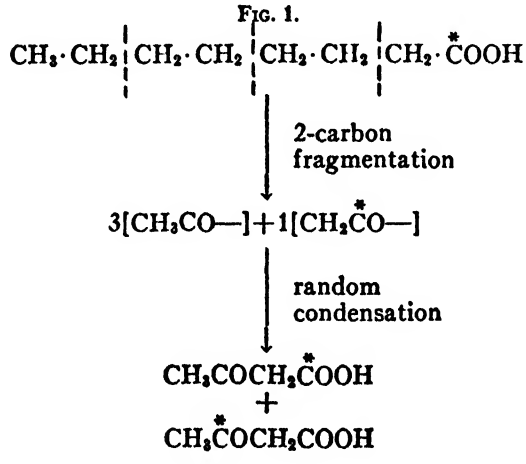
In recent years the concept of four-carbon fragmentation (rather than cleavage producing two-carbon units) received considerable support largely as a result of the experiments of Hurtley (1916), Jowett and Quastel (1935b), Leloir and Munoz (1939), Blixenkrone-Moeller (1939), and Stadie (1941). This theory called multiple alternate oxidation was proposed as the most plausible explana-

tion for the fact that increasing chain length magnifies the yield of ketone bodies from fatty acids, thus suggesting that a very appreciable portion of the fatty acid chain is utilized in the formation of ketone bodies. The evidence supporting this theory has been fully presented by Stadie (1945).

The finding by Jowett and Quastel (1935b), Butts, *et al.* (1935) and Deuel, *et al.* (1936) that hexanoic acid yields more ketone bodies per mole than butyric acid, has resulted in a new concept. MacKay, Wick and Barnum (1940) demonstrated clearly that valeric acid is ketogenic and suggested that the five-carbon chain of valeric acid is degraded to a three-carbon fragment along with a two-carbon unit, and that two of the latter units can combine to form acetoacetate. MacKay, Barnes, Carne and Wick (1940) accordingly suggested the current theory known as beta-oxidation-condensation which states that ketone bodies are formed by the condensation of two-carbon fragments arising from the successive beta oxidation of fatty acids. MacKay (1943) has reviewed this field. A similar theory was proposed and promptly rejected by Jowett and Quastel (1935b) because of their belief that such a two-carbon unit could only be acetate. Since acetate was converted to acetoacetate more slowly than fatty acids upon exposure to liver slices, this theory was discarded. There is now available a considerable body of evidence indicating that the two-carbon unit is not acetate.

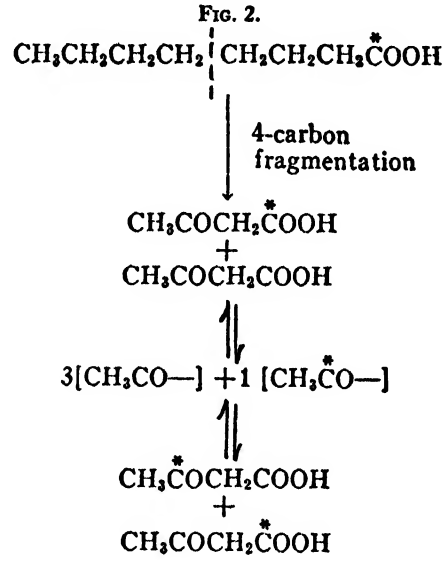
Strong supporting evidence for MacKay's concept was provided by Weinhouse, Medes and Floyd (1944) who synthesized isotopic octanoic acid

FIG. 1.



(carboxyl labeled) and studied the distribution of C^{13} in the acetoacetate formed from the isotopic acid by liver slices (Fig. 1). The acetoacetate formed from the octanoate was found to contain isotope equally distributed between the carbonyl and carboxyl carbons. These results are consistent with the theory of MacKay. If the resulting acetoacetate had been exclusively labeled with isotope in the carboxyl position, the conclusion might well have been reached that acetoacetate was derived exclusively by multiple alternate oxidation (four-carbon fragmentation). Had there been an excess of isotope in the carboxyl position as compared with the carbonyl group, it might have indicated the occurrence of some multiple alternate oxidation. The equal distribution that was obtained was accordingly interpreted to mean that acetoacetate was formed

FIG. 2.



exclusively by two-carbon fragmentation followed by random condensation of these fragments.

However, an equally valid alternative may be considered. It is possible that acetoacetate labeled solely in the carboxyl position was first formed by four-carbon fragmentation and that this substance then rearranged into symmetrically labeled acetoacetate by reversible dissociation into two-carbon units (Fig. 2).

The possibility of rearrangement of singly labeled acetoacetate to give doubly labeled acetoacetate has been tested by Buchanan, Sakami and Gurin (1947) who incubated carboxyl labeled acetoacetate with liver slices under conditions identical with those employed by Weinhouse, *et al.* At the end of the incubation period the acetoacetate still retained its isotope exclusively in the carboxyl position. A similar lack of redistribution was found with carbonyl labeled acetoacetate (Table 1).

Taken together these supplementary isotope experiments constitute proof that two-carbon fragmentation *precedes* the formation of acetoacetate from fatty acids. It is possible, however, that some *other* four-carbon intermediate formed from fatty acids is capable of undergoing reversible dissociation into two-carbon units before being irreversibly changed to acetoacetate. Such an alternative would

account for the results obtained by Medes, Weinhouse and Floyd (1945). In these experiments the conversion of carboxyl labeled butyrate to acetoacetate by liver slices was studied, and it was observed that the resulting acetoacetate contained a significant excess of isotope in the carboxyl as compared to the carbonyl position. This result was interpreted to mean that some cleavage to two-carbon units had occurred along with appreciable direct oxidation

TABLE 1. REACTIONS OF CARBONYL OR CARBOXYL LABELED ACETOACETATE WITH RAT LIVER SLICES

	Carbonyl labeled acetoacetate		Carboxyl labeled acetoacetate	
	mM	C^{13} % excess	mM	C^{13} % excess
Acetoacetate (initial)	0.130		0.130	
Carboxyl carbon		0.00		2.07
Carbonyl carbon		1.20		0.39
Acetoacetate (final)	0.148			
Carboxyl carbon		0.02		1.33
Carbonyl carbon		0.78		0.24

of butyrate to acetoacetate. Since acetoacetate is not cleaved by liver slices to two-carbon intermediates, it is apparent that some other compound derived from butyrate is capable of undergoing this cleavage.

THE COMPLETE OXIDATION OF FATTY ACIDS, KETONE BODIES AND ACETATE

For more than ten years evidence has accumulated suggesting that fatty acids may be oxidized by reactions similar to those of the tricarboxylic acid cycle. In 1935, Quastel and Wheatley (1935) reported that malonate, a poison of the succinoxidase system, is able to inhibit the oxidation of fatty acids and ketone bodies by tissue slices. More direct evidence has come from the laboratories of Sonderhoff and Thomas (1937), Lynen (1942) and Virtanen and Sundman (1942) who have demonstrated that yeast is able to convert acetate into succinate and citrate. Out of this work arose the suggestion that acetate may condense with a four-carbon dicarboxylic acid (presumably oxalacetate) to form citrate. Wieland and Rosenthal (1943) as well as Breusch (1943) have independently reported that extracts or breis of kidney and muscle tissue are capable of forming more citrate from oxalacetate plus acetoacetate (or acetate) than from oxalacetate alone. As previously mentioned, Breusch claims that many beta keto acids can undergo this reaction. Wieland and Rosenthal were able to isolate and characterize the citric acid formed by this reaction in kidney, and postulated a direct condensation of oxalacetate with acetoacetate to form citrate.

Isotopic experiments have aided materially in clarifying this field. Buchanan, Sakami, Gurin and Wilson (1945) found that the aerobic disappearance of acetoacetate in homogenates of guinea pig kidney is stimulated approximately three fold by the addition of any of the organic acids of the tricarboxylic acid cycle. To determine whether or not acetoacetate is oxidized by this pathway, isotopic acetoacetate $CH_3\overset{\bullet}{C}OCH_2\overset{\bullet}{C}OO^-$ was incubated with homogenized kidney cortex in the presence of non-isotopic succinate or α-ketoglutarate. Fumaric acid was isolated in the first instance and α-ketoglutaric acid in the latter case (Table 2). The isolated acids contained a sufficient excess of isotope to indicate that a large portion of the acetoacetate that had disappeared was actually metabolized via the tricarboxylic acid cycle. In a similar experiment it was demonstrated that isotopic acetate $CH_3\overset{\bullet}{C}OO^-$ was converted to isotopic succinate.

By degradation of the isolated acids it was found that the fumarate was labeled solely in the carboxyl groups while the α-ketoglutarate contained approximately ten times as much isotope in the γ carboxyl group as in the α carboxyl carbon. This result agrees with what is well known in the case of pyruvate metabolism and which has been adequately reviewed

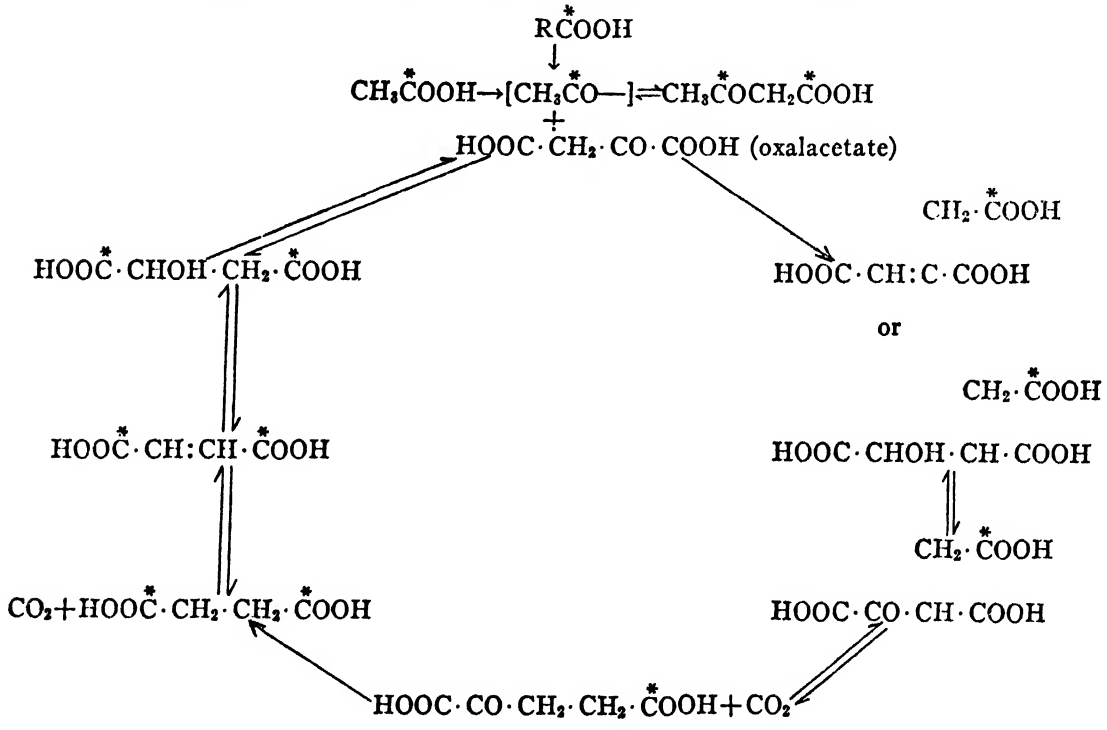
TABLE 2. REACTIONS OF ACETOACETATE AND ACETATE IN KIDNEY HOMOGENATES

Experiment No.	Isotopic material added	Amount of acetoacetate or acetate metabolized	Non-isotopic organic acid added	Dicarboxylic acid isolated	C^{13} concentration, atoms % excess: Initial acetoacetate or acetate	Dicarboxylic acid isolated	$NaHCO_3$ (average value)
(1)	(2)	(3) mM	(4)	(5)	(6)	(7)	(8)
1A	Acetoacetate	0.25	Succinic	Fumaric	3.80	0.22	0.12
1B	Acetoacetate	0.34	Succinic	Fumaric	3.98	0.30	0.01
2	Acetoacetate	0.32	α-Ketoglutaric	α-Ketoglutaric	3.98	0.50	0.03
3	CO_2	0.10	Succinic	Fumaric	0.00	0.00	2.28
4	Acetate	0.31	Acetoacetic α-Ketoglutaric	Succinic	3.88	0.28	0.01

elsewhere (Buchanan and Hastings, 1946; Wood, 1946), namely, that citric acid is not a direct product of the oxidation of acetoacetate or acetate. The nature of the initial condensation reaction in mammalian tissue is still unknown, nor is there conclusive information regarding the identity of the product formed.

Weinhouse, *et al.* (1945a) successfully isolated isotopic α-ketoglutarate from rat kidney mince incubated with labeled acetate. In confirmation of the above-mentioned results evidence was obtained suggesting that the isotope was located predominantly

FIG. 3. Oxidation of Fatty Acids, Acetoacetate, and Acetate by the Tricarboxylic Acid Cycle.



in the γ carboxyl position. Rittenberg and Bloch (1945) after feeding carboxyl labeled acetate to rats and mice succeeded in isolating glutamic and aspartic acid which were found to contain excess isotope. Since these amino acids are in equilibrium with α-ketoglutarate and oxalacetate, these results add further proof for the concept that acetate is metabolized via the tricarboxylic cycle (Fig. 3). The details of this cycle have been reviewed elsewhere (Buchanan and Hastings, 1946; Wood, 1946; Gurin, 1948).

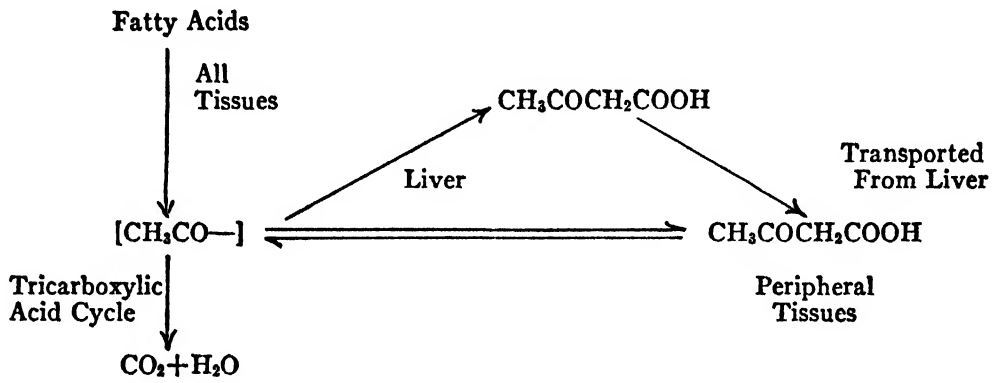
Krebs and Eggleston (1948), employing non-isotopic techniques, have agreed that, under aerobic conditions, acetoacetate is oxidized by way of the tricarboxylic acid cycle. Furthermore these investigators have confirmed the fact that citrate is formed primarily by a side-reaction and that some other tricarboxylic acid is involved in this sequence of reactions.

Although it is clear that acetoacetate, as well as acetate, is converted to carbon dioxide and water by way of the tricarboxylic acid cycle, there are still many questions that can be raised. Medes, Floyd and Weinhouse (1946) have shown that acetoacetate is not a direct intermediate arising from the oxidation of acetate by kidney. Acetoacetate is, therefore, probably not the substance undergoing direct condensation with oxalacetate. Buchanan, *et al.* (1947) incubated isotopic acetoacetate with non-isotopic acetate and α-ketoglutarate in rabbit kidney extracts. The acetate isolated at the end of the experiment contained much less isotope than did the α-ketoglutarate. Acetoacetate is, therefore, converted via the tricarboxylic cycle to α-ketoglutarate without forming acetate as an intermediate. Although acetate is not a direct intermediate, it is probable that some other two-carbon fragment is involved. Upon incubation with kidney slices, singly labeled acetoacetate redistributes its isotope to a significant extent—a result which can best be explained by reversible dissociation into two-carbon units (Buchanan, Sakami and Gurin, 1947).

It has previously been noted that Lehninger obtained evidence indicating that in the presence of

four-carbon dicarboxylic acids, fatty acids can be oxidized while acetoacetate is not affected by this system. Neither acetate nor acetoacetate can, therefore, be considered to be important intermediates in the oxidation of fatty acids via the tricarboxylic cycle. The acetoacetate that is formed in liver is probably transported elsewhere to be converted to a two-carbon intermediate (not acetate) prior to condensation with oxalacetate. In this connection it is of interest that Hunter and Leloir (1945) have demonstrated that in kidney cortex, acetoacetate undergoes some preliminary reaction (conversion to a two-carbon intermediate ?) which is facilitated by oxidation of α-ketoglutarate before condensation with oxalacetate to form a tricarboxylic acid is accomplished. Lehninger (1946a) has also concluded that acetoacetate is not the condensing agent in citrate formation but rather that some two-carbon intermediate is involved.

Fig. 4.



Fatty acids are, therefore, cleaved into two-carbon fragments in liver and probably in extrahepatic tissues (Fig. 4). In the liver these two-carbon fragments may presumably be (1) oxidized to a limited extent (2) converted to acetoacetate which may be considered to act as a reservoir for 2-carbon units. These fragments are subsequently transported as such or as acetoacetate to extrahepatic tissues. In peripheral tissue it can be presumed that the two-carbon fragments derived from fatty acids or from acetoacetate (Buchanan, Sakami and Gurin, 1947) are promptly oxidized via the tricarboxylic acid cycle.

Reference to Figure 3 will indicate the disposition of isotope via the oxidative cycle. The over-all reaction is essentially 1 $CH_3COOH \rightarrow 2\ CO_2 + 2\ H_2O$. It will be noted, however, that the two carbon atoms eliminated as CO_2 are derived not from the two-carbon intermediate (representing fat) but from the oxalacetate necessary for the initial condensation. This scheme illustrates how acetic, acetoacetic and fatty acids may be oxidized by a mechanism common to carbohydrate oxidation without effecting a net synthesis of carbohydrate. The cycle is completed without the accumulation of any more carbohydrate precursor (oxalacetate) than was initially present to start the cycle.

Studies on the Nature of the Two-Carbon Intermediate

There have been numerous reports indicating that liver slices (Annau, 1934; Edson, 1935; Krebs and Johnson, 1937) and homogenates (Lehninger, 1946a) are capable of transforming pyruvate into acetoacetate. Crandall, Gurin and Wilson (1947) have demonstrated that α,β labeled pyruvate is converted to completely labeled acetoacetate upon incubation with washed liver homogenates. Further indication that pyruvate is capable of supplying its alpha and beta carbons for the synthesis of acetoacetate was obtained by the administration of α,β labeled lactate to phlorhizinized rats (Gurin, Delluva and Wilson, 1947). The resulting urinary acetoacetate contained isotope distributed equally among all four of its carbon atoms.

That there is a close parallelism between the behavior of pyruvate and fatty acids with regard to the formation of acetoacetate as well as intermediates of the tricarboxylic acid cycle has been established by Lehninger (1946a). This investigator has demonstrated that pyruvate or octanoate can be quantitatively converted to acetoacetate by washed homogenates of rat liver. Furthermore, the addition of a four-carbon dicarboxylic acid to the medium (as a source of oxalacetate) diverts both the pyruvate and octanoate from the production of acetoacetate to the formation of intermediates of the tricarboxylic acid cycle.

This washed centrifugate of homogenized liver, because of its restricted metabolism, has provided a useful and valuable means of testing the ability of various substances to act as intermediates in the synthesis of acetoacetate or of members of the tri-

carboxylic acid cycle. Apparently the main pathways of fatty acid and pyruvate oxidation have remained intact in this preparation while side reactions have been eliminated. Acetate is known to form ketone bodies readily in the presence of liver slices and has therefore in the past been seriously considered as a possible 2-carbon intermediate. In the washed homogenate, however, Lehninger (1946a) has shown acetate to be inactive as a source of acetoacetate, and it is therefore eliminated as an intermediate in the conversion of either pyruvate or fatty acids to acetoacetate.

He has also tested a series of two-carbon compounds including acetate, acetaldehyde, glycollate, glyoxylate, oxalate, ethanol, acetamide, glycine, acetyl phosphate and acetyl pyrophosphate as precursors of acetoacetate in this system. All were reported to be negative (Lehninger, 1946a). Acetaldehyde, however, can form acetoacetate at an appreciable rate although this conversion is always slower than that given by octanoate (Lehninger, personal communication). When these substances as well as acetyl glycine, acetyl choline and acetyl p-amino benzoate were tested for citrate formation in the presence of a source of oxalacetate, only acetate produced citrate in significant quantities. The yield of citrate from acetate was substantial and approached that obtained with octanoate (Lehninger, personal communication).

It seems likely that both acetaldehyde and acetic acid may be closely related to the two-carbon intermediate. These preliminary results also suggest that the two-carbon precursor of acetoacetate and the two-carbon compound which condenses with oxalacetate to form citrate may be different substances. In both instances, the presence of adenosine triphosphate was obligatory, suggesting that both acetaldehyde and acetate may have been converted to phosphorylated intermediates as a prerequisite to activity. It should be noted also that the adenylic system is required for the oxidation of pyruvate and fatty acids in the washed liver homogenate (Lehninger and Kennedy, 1948).

It is of interest that Lehninger's experiments (1945) also eliminated crotonic, vinylacetic, DL-β-hydroxybutyric and acetopyruvic acids as important intermediates in the formation of acetoacetate.

Isotopic evidence that acetate is not an intermediate in the formation of acetoacetate was also obtained by Weinhouse, *et al.* (1945b) who incubated carboxyl labeled acetate with liver slices. In these experiments it was found by observing the dilution of isotope in the resulting acetoacetate, that 50 percent of it arose endogenously from a non-isotopic source. The isotope concentration of the recovered acetate, however, remained unchanged throughout the experiment indicating no production of acetate while endogenous acetoacetate was being formed. Mention has already been made of the evidence demonstrating that isotopic acetoacetate is converted by extracts of kidney to α-ketoglutarate without intermediate formation of acetate. Although the two-carbon intermediate is not acetate, it is probably capable of secondarily forming acetate or entering into equilibrium with it to some extent.

The simplest explanation for the parallel behavior of pyruvate and octanoate in the washed liver homogenate is that pyruvate and octanoate both give rise to the same two-carbon intermediates. However, there is evidence to the contrary which has recently been reviewed by Bloch (1947). Preliminary experiments with acetate, in agreement with Lehninger's, showed acetate to be quite incapable of forming acetoacetate in the washed homogenate (Crandall, Gurin and Wilson, 1947). In these experiments carboxyl labeled acetate and a small sample of non-isotopic acetoacetate were incubated together in the homogenate. The acetoacetate recovered at the end of the incubation period was found to contain no isotope. When carboxyl labeled acetate was incubated with non-isotopic pyruvate, however, a small but significant amount of isotope appeared consistently in the resulting acetoacetate (Table 3).

TABLE 3. ACETOACETATE FORMATION FROM CARBOXYL-LABELED ACETATE AND NON-ISOTOPIC PYRUVATE (6 Atom % excess C^{13})

Initial Concentrations of:			Atom % excess C^{13} in final Acetoacetate		
Pyruvate	Acetate $\overset{*}{C}OOH$	Aceto-acetate	$\overset{*}{C}O$	$\overset{*}{C}OOH$	$\frac{\overset{*}{C}O}{\overset{*}{C}OOH}$
0.008 M	0.001 M		0.30	0.30	1.00
	0.001	0.0022 M	0.00	0.03	
0.015	0.006		0.39	0.35	1.10
	0.006	0.0031	0.00	0.03	
0.015	0.006		0.36	0.39	0.93
0.015	0.020		0.21	0.23	0.91

The location of the isotope exclusively in the carboxyl position would have indicated that the utilization of acetate depended entirely upon its being acetylated by pyruvate. Conversely, the formation of purely carbonyl labeled acetoacetate would have indicated the acetylation of pyruvate by acetate or one of its metabolic derivatives. The isotope was found, however, to be equally distributed between both the carbonyl and carboxyl carbons in all such experiments. It was, therefore, impossible to decide whether acetate had condensed interchangeably with fragments derived from pyruvate or

TABLE 4. ACETOACETATE FORMATION FROM CARBOXYL-LABELED ACETATE AND NON-ISOTOPIC OCTANOATE (6.0 Atom % excess C^{13})

Initial Concentrations of:			Atom % excess C^{13} in final acetoacetate		
Octanoate	Acetate $\overset{*}{(COOH)}$	Aceto-acetate	$\overset{*}{CO}$	$\overset{*}{COOH}$	$\frac{\overset{*}{CO}}{\overset{*}{COOH}}$
0.001 M	0.002 M		0.27	0.50	0.54
	0.002	0.0031 M	0.00	0.04	
0.001	0.004		0.51	0.66	
	0.004	0.0029	0.28	0.27	
			0.23	0.39	0.59

whether it was merely converted into a reactive fragment as a result of the energy supplied by the simultaneous oxidation of pyruvate.

When similar experiments (Crandall, Gurin and Wilson, 1948) were carried out with carboxyl labeled acetate and non-isotopic octanoate, again a similar small but significant incorporation of acetate carbon into acetoacetate was observed (Table 4). However, in this case, the distribution of isotope between the carbonyl and carboxyl carbons was uneven: nearly a two fold excess appearing in the carboxyl position. According to this result at least 50 percent of the isotopic acetoacetate formed must have been purely carboxyl labeled. Here the production of this purely carboxyl labeled component of the mixed acetoacetate must have arisen by the acetylation of carboxyl labeled acetate molecules by non-isotopic two-carbon fragments derived from octanoate.

The differences between the effect of pyruvate and octanoate upon the incorporation of acetate into acetoacetate may be taken as evidence that pyruvate and octanoate give rise to different two-carbon intermediates.

TABLE 5. EXPERIMENTS WITH CARBOXYL LABELLED OCTANOATE USING RAT LIVER SLICES

	Experiment 1		Experiment 2	
	mM	C^{13} %	mM	C^{13} %
Octanoate (initial)	0.400		0.400	
Carboxyl carbon		7.68		7.68
Acetoacetate (initial)	0.107		0.100	
Carboxyl carbon		0.00		0.00
Carbonyl carbon		0.00		0.00
Acetoacetate (final)	0.110		0.175	
Carboxyl carbon		0.91		0.98
Carbonyl carbon		0.63		0.60
Total ketones	0.150		—	

In the previously mentioned experiments of Weinhouse, *et al.* (1945b) in which carboxyl labeled acetate was incubated with liver slices, the isotopic acetoacetate formed was found to contain a less pronounced but definite asymmetry of the same type we observed in liver homogenates. It would appear as if liver slices were blending symmetrically labeled acetoacetate (made from acetate in the presence of pyruvate) with the asymmetrically labeled type formed in the presence of metabolizing fatty acids. It should be emphasized, however, that the incorporation of isotopic acetate into acetoacetate was much greater in the liver slices than in the homogenates.

There is further evidence which suggests not only that pyruvate and octanoate may give rise to different two-carbon intermediates, but that an aliphatic acid may itself conceivably give rise to more than one type of two-carbon intermediate. In an attempt to repeat the experiments of Weinhouse, Medes, and Floyd (1944) in which liver slices were incubated with carboxyl labeled octanoate, Buchanan, Sakami and Gurin (1947) failed to obtain the symmetrically labeled acetoacetate reported by

TABLE 6. ACETOACETATE FORMATION FROM CARBOXYL-LABELED OCTANOATE IN WASHED LIVER HOMOGENATE

Octanoate		Atom % excess C^{13} in final acetoacetate		
Atom % excess C^{13}	Initial conc. in medium	$\overset{*}{CO}$	$\overset{*}{COOH}$	$\frac{\overset{*}{CO}}{\overset{*}{COOH}}$
7.68	0.001 M	1.32	2.01	0.66
7.68	0.001	1.47	1.79	0.82
23.8	0.001	3.72	6.64	0.56
23.8	0.001	4 17	6.07	0.69
23.8	0.001	3.45	6.05	0.57
23.8	0.001	3.84	5.92	0.65

Weinhouse. Twice as much isotope was found in the carboxyl as was in the carbonyl carbon, suggesting at that time the occurrence of some multiple alternate oxidation in these experiments (Table 5). Subsequent experiments (Crandall, Gurin and Wilson, 1947) in which carboxyl labeled octanoate was incubated in the washed liver homogenate gave essentially the same results (Table 6). In every case the resulting acetoacetate contained significantly more isotope in the carboxyl than in the carbonyl position. Carboxyl labeled hexanoic acid reacts in exactly the same way. As previously mentioned, Weinhouse has obtained similar results with carboxyl labeled butyrate. If the asymmetry observed here is to be ascribed to the occurrence of a significant amount of multiple alternate oxidation (four-carbon fragmentation), experiments with octanoic acid labeled in the beta position should lead to the formation of acetoacetate with the asymmetry

reversed, that is, with twice as much isotope in the carbonyl as in the carboxyl position. However, this was not the case (Table 7). The results obtained with beta-labeled octanoate were essentially the same as those obtained with carboxyl-labeled octanoate (Crandall, Gurin and Wilson, 1948). These findings eliminate multiple alternate oxidation either as an explanation for the asymmetrical distribution of isotope in these experiments, or as a mechanism necessary for the oxidation of fatty acids by liver.

It is possible that the discrepancy between these results and those of Weinhouse, Medes and Floyd may be due in part to differences in the techniques used in the isolation and degradation of acetoacetate in the two laboratories. It is difficult on this basis, however, to explain the symmetrical distribution of isotope in acetoacetate arising from α,β-labeled pyruvate under the same experimental conditions.

A theoretical explanation of the unequal distribution of isotope in this acetoacetate involves the

TABLE 7. ACETOACETATE FORMATION FROM β-LABELED OCTANOATE IN WASHED LIVER HOMOGENATE

Octanoate		Atom % excess C^{13} in final acetoacetate		
Atom % excess C^{13}	Initial conc. in medium	$\overset{*}{CO}$	$\overset{*}{COOH}$	$\frac{\overset{*}{CO}}{\overset{*}{COOH}}$
4.8	0.001 M	0.63	1.36	0.46
4.8	0.001	0.63	1.19	0.53

possibility that octanoate can give rise to two types of two-carbon fragments with the result that condensation of these fragments to form acetoacetate is not as completely random as would be expected between fragments which are all alike. This could lead to the observed asymmetrical isotope distribution of acetoacetate. According to this hypothesis the two-carbon fragment derived from the carboxyl and alpha carbons of octanoate and the second fragment derived from the beta and gamma carbons must be similar since beta-labeled, as well as carboxyl labeled octanoate both give rise to labeled acetoacetate with the same distribution of isotope. One would expect the third fragment to be identical with the first two, so that the fourth or terminal fragment (containing a methyl group rather than methylene) must be the one that is different from the others if the assumption is correct that differences exist. According to this hypothesis, terminally labeled octanoic acid should give rise to acetoacetate with the asymmetry reversed. This hypothesis can also explain the pronounced asymmetry observed in the acetoacetate formed from butyrate (Weinhouse) since a short chain fatty acid contains a relatively great proportion of the terminal fragment.

TABLE 8. ACETOACETATE FORMATION FROM CARBOXYL-LABELED OCTANOATE IN THE PRESENCE OF NON-ISOTOPIC PYRUVATE

Octanoate		Pyruvate Initial conc. in medium	Atom % excess C^{13} in final acetoacetate		
Atom % excess C^{13}	Initial conc. in medium		$\overset{*}{CO}$	$\overset{*}{COOH}$	$\frac{\overset{*}{CO}}{\overset{*}{COOH}}$
7.68	0.001 M	0.004	0.84	0.84	1.00
7.68	0.001	0.004	0.87	1.01	0.86†
23.8	0.001	0.008	1.62	1.99	0.81†
23.8	0.001	0.008	1.86	2.28	0.82†

† Synthetic acetoacetate containing equal concentrations of C^{13} in the carbonyl and carboxyl positions gave similar CO/COOH values by our methods of analysis.

To return to the question of the production of different types of two-carbon intermediates by pyruvate and octanoate during formation of acetoacetate, additional evidence for this has been obtained by incubating carboxyl-labeled octanoate with non-isotopic pyruvate in the washed homogenate (Table 8). The distribution of isotope in the acetoacetate which is formed under these conditions is significantly different from that obtained when carboxyl labeled octanoate is incubated alone (compare with Table 6). A consistent increase in the symmetry of distribution is observed whenever either carboxyl labeled or beta labeled octanoate is incubated with non-isotopic pyruvate (Table 9; compare with Table 7). Although it is impossible to draw specific conclusions from these observations alone, they do indicate that pyruvate yields intermediates which interact with metabolites arising from octanoate in such a way as to alter the distribution of isotope in the mixed acetoacetate. It

TABLE 9. ACETOACETATE FORMATION FROM β-LABELED OCTANOATE IN THE PRESENCE OF NON-ISOTOPIC PYRUVATE

Octanoate		Pyruvate Initial conc. in medium	Atom % excess C^{13} in final acetoacetate		
Atom % excess C^{13}	Initial conc. in medium		$\overset{*}{CO}$	$\overset{*}{COOH}$	$\frac{\overset{*}{CO}}{\overset{*}{COOH}}$
4.8	0.0005 M	0.004	0.33	0.39	0.85†
4.8	0.0005	0.002	0.48	0.55	0.87†

† See footnote to table 8.

follows from this that pyruvate must give rise to a two-carbon intermediate or mixture of intermediates which differ qualitatively from those produced by octanoate. If both substances were capable of giving rise to the same metabolic mixture, then the distribution of isotope in the acetoacetate would have remained unchanged.

It is apparent that much remains to be accomplished in this field before the nature of the two-carbon fragments, and the identity of the intermediary metabolites derived from the catabolism of fatty acids are well understood. Isotopic techniques will undoubtedly continue to play a leading role in such developments. It is also evident that separation of individual enzyme systems will be a primary requisite for the ultimate solution of many of these problems.

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DISCUSSION

LIPMANN: Dr. Gurin concluded his interesting talk with the remarks that complementary work with isolated enzyme systems will eventually throw light on the finer chemical mechanisms involved. Since a large part of the present talk centered around incorporation of marked acetate into acetoacetate, it appears appropriate, as a contribution in the indicated direction, to report here our recent results on a net synthesis of acetoacetate from acetate with an isolated enzyme system—namely, pigeon liver extract.

During work on the activation of acetate with ATP in liver extract, it was consistently observed that the addition of acetate alone caused an increased dephosphorylation of ATP. This breakdown was enhanced by the addition of hydroxylamine or sulfanilamide as acetyl acceptor, when acethydroxamic acid or acetyl sulfanilamide was formed. These observations made us suspect that acetate itself might, in this extract likewise, act with its methyl part as acetyl acceptor and form thus acetoacetate. Dr. Morris Soodak and I have verified the suspected reaction with pigeon liver extract as well as with ammonium sulfate fractions made thereof. The yields are relatively small, but with two different techniques for acetone determination (Behre; Greenberg and Lester), and, in a few experiments, with the manometric aniline method for acetoacetate, we have found the formation of acetoacetate from acetate +ATP of around 3 μm per cc. of extract. Like other acetylation reactions, the acetoacetate synthesis is dependent on the presence of coenzyme A.

The analogy between acetoacetate formation and other acetylations suggests that a carboxyl-activated acetate reacts with the methyl group of another acetate. We are then dealing here with two differently reacting molecules of acetate:

$$\begin{array}{c} \text{tail}_1 \,.\, \text{head}_1 \rightarrow \text{tail}_2 \,.\, \text{head}_2 \\ CH_3 \,.\, COOH + CH_3 \,.\, COOH \\ + \sim \text{ph} \downarrow \\ CH_3 \,.\, CO \,.\, CH_2 \,.\, COOH + \text{ph} \end{array}$$

In our laboratory slang, we like to talk about a head and tail of acetic acid. The carboxyl end, we call the head, the methyl end, the tail of the acetic acid. The energy-requiring reaction is, apparently, a head or carboxyl activation, while the methyl end acts as a more or less passive acceptor for the activated brother molecule. In acetoacetate synthesis, obviously, the activated carboxyl is converted to carbonyl; while the eventual carboxyl of acetoacetate belonged previously to the passive acceptor-acetate. Such consideration may be helpful in a discussion of the differences observed in the carbonyl-carboxyl distribution in acetoacetate when formed from carboxyl-labelled acetic acid with non-labelled acetyl donors such as pyruvate or octanoate.

Enlarging still a little more on the distinction between head and tail reaction of acetate, it may be remembered that the entry of acetate into the citric acid cycle is essentially a methyl, or tail, reaction. It seems important to know if and how the tail reaction of acetate in acetoacetate synthesis and that in "citric" acid synthesis are related to each other.

We should like to turn now for a few moments to discussing the nature of the reaction product of acetate with ATP which is, apparently, an acetyl

precursor. (Acetate + ATP) has now been identified as acetyl donor in a variety of reactions. But it is true that synthetic monacetyl phosphate was inactive as a donor in enzyme systems of this type. Now Dr. Nathan O. Kaplan has, in our laboratory, made promising progress towards the identification of what may be called, the "natural" acetyl phosphate. It was found that in extracts of dried *E. coli*, a reaction product of acetate with ATP accumulates which contains equivalent amounts of labile phosphate and reactive acetyl (hydroxamic acid reaction) like synthetic acetyl monophosphate but differing in some respects from the synthetic product:

(1) It is more stable to the muscle enzyme, acetyl phosphatase.

(2) It transfers more sluggishly phosphate to ADP.

And (3) with *E. coli* extract, the "natural" compound will act as acetyl donor to formic acid, yielding pyruvic acid without addition of ATP. The further identification of the acetyl donor molecule, possibly an acetyl phosphate derivative, is now in progress.

HISTORICAL SKETCH OF THE BIOLOGICAL APPLICATION OF TRACER ELEMENTS

G. HEVESY

Faced with the task of giving a survey of the historical development of the application of tracer elements in the field of biology, my first thoughts are with that great man, the late Lord Rutherford, the founder of nuclear sciences, to whom we owe directly or indirectly so much of the development in recent decades. Lord Rutherford used to pull the leg of the chemist in the genial and friendly way in which the Cambridge man teases the Oxford man. One of the reasons which induced him chattily to criticise the chemists was their inability to separate the precious radium D which was imbedded in the large amounts of lead obtained in the separation of radium from pitchblende. Rutherford's institute was in those years interested mainly in the study of the radiation emitted by radioactive bodies, and the huge amounts of lead with which radium D was combined frustrated the use for radiation studies of this precious material present in the hundreds of kilograms of radiolead generously presented to Lord Rutherford by the Vienna Academy of Sciences.

In 1911 and 1912, the author tried in Rutherford's laboratory to effect a separation of radium D from lead, and the failure to obtain a positive result gave him the idea of making use of the inseparability of radium D from lead as a means of labeling lead salt by adding radium D (easily obtainable from old tubes which once contained radium emanation, or from radium salt samples not freshly prepared) to lead salts, and to follow the path of the labeled lead atoms by making use of radioactive measurements.

We in Manchester were not the only ones interested in the separation of radium D from lead. The Vienna Institute for Radium Research disposed of huge amounts of lead chloride prepared from pitchblende and Dr. Paneth, then associate of that institute, made very extended but abortive attempts to obtain a separation of radium D from lead. He failed as well. We became associated late in 1912, making use of the then unique resources of the Vienna Institute to label lead sulfide and lead chromate with radium D and to apply these labeled compounds in the determination of their solubility (Hevesy and Paneth, 1913). It would be tempting here to describe some of the work done in those early years with labeled lead and bismuth. I shall, however, confine myself to a discussion of only one of these investigations, the results of which proved later to be of some importance in the biological application of tracer elements.

Shortly after the end of the first world war, Zechmeister and myself (Hevesy and Zechmeister, 1920) prepared labeled plumboacetate, dissolved equivalent amounts of this salt and of tetraphenyl lead in amyl alcohol, and separated the two compounds by crystallization. The tetraphenyl lead was found to be inactive, demonstrating the lack of interchange between the lead atom of tetraphenyl lead and lead ions. Other organic lead compounds investigated have shown the same absence of interchange. Between the lead atoms of formiate and lead acetate or other compounds containing dissociating lead ions, prompt interchange was found to take place. These early experiments indicated the lack of interchange of atoms present in organic binding, a result which was confirmed by later experiments in which labeled phosphorus, sulfur etc. were brought together with organic compounds present in the organism. After shaking, for example, a solution of glycerophosphate (Perrier and Segré, 1938), lecithin, casein (Aten, 1939) or desoxyribosenucleic acid (Hahn and Hevesy, 1940; Born *et al.*, 1941) with a solution of labeled phosphate, no organic labeled phosphorus was found to be present. Exchanges were found to occur—to consider another element—between molecular iodine and diiodotyrosine and also between iodide and diiodotyrosine at pH 4.3, not however, at a pH which prevails in the animal organism (Miller *et al.*, 1944). The above statement of non-interchange does not apply to hydrogen bound to oxygen or nitrogen (Reitz and Bonhoeffer, 1934) which has an unique position among the elements.

The question of to what extent atoms incorporated in organic molecules interchange with atoms present in other molecules or ions is of great importance for the applicability of isotopic indicators to biological research. We use isotopic tracers in the determination of rate and place of formation of biochemically important compounds in the organism, and try to elucidate the mechanism of the enzymatic processes leading to the formation of compounds such as phosphatides, nucleic acid, glycogen, methionine, and so on. All these determinations are based on the assumption that the tracer atoms, ions or radicals do not interchange with the corresponding constituents of the molecules in question. The administered labeled phosphate is assumed not to exchange with the phosphate radical of the phosphatide molecule, for example, by any simple "physical" exchange process. The formation of labeled phosphatides by collision between an inorganic phosphate molecule and a phosphatide molecule, solely as a result of such a collision, is assumed not to take place. The formation of labeled phosphatide mole-

cules in the organism takes place as a result of an enzymatic process which is coupled with an energy-producing system. These conclusions are strongly supported by the results of experiments carried out by Chaikoff and his associates. They found that respiratory inhibitors (Morton and Chaikoff, 1943) or anaerobic conditions (Taurog *et al.*, 1942) block the formation of tagged phosphatides in tissue slices which are incubated in Ringer solution containing labeled phosphate.

Earliest Biological Application

In contradistinction to the animal body, the uptake of mineral constituents by the plant is not followed by a loss of such constituents, and it was formerly considered that the ions taken up by the roots of the plant did not pass in the opposite direction at all. The application of isotopic indicators, however, has shown that this is not the case. Ions taken up by the plant can be removed by an exchange process under the action of other ions present in the soil or in the nutrient solution. It had already been found in 1923 that minute amounts of lead, labeled by the admixture of the lead isotope thorium B, when taken up by the roots of *Vicia faba,* could to a large extent be removed by an excess of non-labeled lead added to the nutrient solution (Hevesy, 1923). Most other ions were found to be much less effective in removing the labeled lead ions from the plant.

In recent years, the behavior of essential constituents of plants has been investigated, making use of artificial, radioactive ions as indicators; similar results have been obtained. Mullins and Brooks (1939) placed cells of *Nitella coronata,* first in a solution containing radioactive potassium, and later in solutions of different chlorides. Sodium and lithium were found to be much less effective in removing labeled potassium than potassium itself, whereas rubidium was more effective. Jenny and Overstreet (1939) and Broyer and Overstreet (1940) found that ionic exchange could take place during periods of, and under conditions favorable for, active solute uptake.

In the early twentieth century, dermatologists became interested in the replacement of arsenic compounds by bismuth compounds in syphilis therapy. To obtain data on the resorbability, distribution and excretion of various preparations containing bismuth, labeled bismuth compounds were administered to rats. This was the first application of radioactive indicators to the study of animal metabolism (Christiansen *et al.*, 1924a) and was soon followed by similar studies using labeled lead (Christiansen *et al.*, 1924b; Behrens, 1925).

The success achieved by Blair-Bell in cancer therapy using lead compounds induced the investigation of the partition of labeled lead between normal and tumor tissue (Hevesy and Wagner, 1930). Though this work gave a negative result, it nevertheless proved to be of great importance in the future development of isotopic indicators. The investigation of the partition of lead was carried out in the Institute of Physical Chemistry of the University of Freiburg. Feeling the need of an assistant trained in biology, I approached Dr. Aschoff, the director of the Institute of Pathology. He selected a Japanese scientist for this work, who however proved to have difficulties in coping with the task. It was then that Dr. Schoenheimer, associate of the Institute of Pathology, was asked by Dr. Aschoff to step in. It was in the course of these investigations that Schoenheimer became familiar with the method of isotopic indicators, which he applied several years later with such great success in the study of fat and protein metabolism and of numerous related problems. Never were more beautiful investigations carried out with isotopic indicators than those of the late Professor Schoenheimer, whose untimely and tragic death is greatly to be deplored.

Importance of the Discovery of Deuterium

The application of natural radioactive bodies as indicators in biology had limited, though not yet exhausted, possibilities. Great things were, however, to come in the following years which made every element available for tracer work. The first step in these events was the discovery by Urey in 1931 of deuterium. This discovery, of very great importance, was bound to impress those formerly engaged in tracer work. Shortly after the announcement of Urey's discovery, studies were initiated of water circulation in the animal organism through the use of dilute heavy water as a tracer. Interchange between the water content of goldfish and the water of their surroundings was found to take place at a remarkable rate, decreasing to some extent with increasing size of the fish and decreasing very substantially after death (Hevesy and Hofer, 1934a). The "average life" of the water molecules drunk by humans was found to be a fortnight (Hevesy and Hofer, 1934b). Later investigations of the rate of the mixture of administered water with the water present in the body of rabbits (Hevesy and Jacobsen, 1940; Hahn and Hevesy, 1941) and guinea pigs (Flexner *et al.*, 1942) led to the result that, while the water reaching the circulation enters into exchange equilibrium with the extracellular water very rapidly, it takes some minutes before equal distribution of the labeled molecules between cellular and extracellular water is reached. In the guinea pig, 73 percent of the water in the blood is exchanged for extracellular water every minute. In early investigations (Hevesy and Hofer, 1934b), the total water content of the human body was calculated from the dilution of the heavy water administered to amount to 63 percent of the body weight, the same considerations being applied by McDougall *et al.* (1934) in the determination of the body water of the rat. The discovery of tritium made it possible

in recent years to employ radioactive (tritiated) water in such experiments (Pace *et al.*, 1947). By this means Krogh and Ussing (1937) demonstrated the impermeability of trout eggs to water during the first stages of their development.

Shortly after the discovery of heavy hydrogen, Bonhoeffer and his colleagues employed deuterium to study the incorporation of this isotope into algae and other growing organisms. The deuterium incorporated in algae was found to interchange with the hydrogen atoms of the nutrient solution only to a restricted extent (Reitz and Bonhoeffer, 1934). In numerous later investigations by Bonhoeffer and his associates (*cf.* Gunther and Bonhoeffer, 1939), the difference in the rate of incorporation of hydrogen and deuterium in growing organisms was mainly studied. Appreciable differences were found. Deuterium is not an ideal indicator, its properties differing appreciably from those of hydrogen, and it must be used with discretion, a fact which, however, did not prevent the attainment of most important results with this tracer element.

In order to exploit the availability of deuterium for the development of biological research, the Rockefeller Foundation established a fund to enable chemists trained in deuterium techniques to apply their special knowledge to biochemical and allied problems. Under these auspices, David Rittenberg came from Urey's group to the Department of Biochemistry of Columbia University, where Schoenheimer was at that time engaged in the investigation of sterol metabolism. From their most happy association developed the idea of employing compounds labeled with deuterium for the study of problems of intermediary metabolism. Their first paper was published in 1935 (Schoenheimer and Rittenberg, 1935a); it describes, among other things, the preparation of stearic acid 9, 10, 12, 13 d_4. The same issue of the *Journal of Biological Chemistry* contained three other papers of these experimenters, one describing methods of the generation of deuterium gas, the combustion of deuterated organic substances, and the determination of the deuterium content in such samples (Rittenberg and Schoenheimer, 1935); the second paper (Schoenheimer and Rittenberg, 1935b) dealing with the fate of the dietary fat in the body, wherein the authors arrive at the important conclusion that the larger part of the dietary fat, even when present in small quantities, is deposited in the fatty tissues before it is utilized; and the third paper (Schoenheimer *et al.*, 1935), in which the conversion of coprostanone into coprosterol in the organism was followed with the help of deuterium.

Numerous following papers (*cf.* Schoenheimer and Rittenberg, 1936, and Schoenheimer, 1942) contain important contributions to the study of fat metabolism. When adult mice were injected with heavy water and given drinking water of such isotopic composition that the deuterium content of the body fluids remained constant throughout the experimental period, half of the fatty acid molecules reached the deuterium level of the body water within a few days. (The half-life time of the regeneration of the deposited fatty acid of the rat was later found by Bernhard and Bullet, 1943, to amount to 9 days.) The biological reversibility of the process was shown in an experiment in which mice previously fed with deuterio fat were placed on a similar diet, but received ordinary water to drink. After the feeding of labeled fat had been discontinued, the isotope level in the total fatty acids of the animals fell at a rate almost identical with that found for the synthesis (Schoenheimer and Rittenberg, 1936). The renewal of fatty acid molecules going on constantly in the organism, a conspicuous example of the dynamic state of body constituents, was thus demonstrated. Rittenberg and Schoenheimer have shown that the synthetic processes are not confined to the fatty acids. Renewal of half the cholesterol content of the mice was found to take place in about three weeks (Rittenberg and Schoenheimer, 1937). In a later investigation, Bloch and Rittenberg (1942) found that, when acetic acid containing deuterium was fed to rats and mice, large amounts of deuterium were found in the cholesterol isolated from the animals. It was thus shown that the organism is able to synthesize cholesterol by condensing acetate molecules.

Smith *et al.* (1936), Stekol and Hamill (1937), Krogh and Ussing (1938), and Ussing (1938) measured the extent of replacement of protein hydrogen in the organs of the mouse by deuterium. The Schoenheimer group extended their investigations at that time (Foster *et al.*, 1938a) to the study of the uptake of deuterium by the amino acids isolated from the organs of rats. All the amino acids, with the exception of lysine, were found to contain considerable amounts of stably bound deuterium. Schoenheimer and his colleagues concluded that this finding must be the result of chemical reactions in which even indispensable amino acids, except lysine, take part and they suggested the occurrence of continuous deamination and amination. Experiments carried out later by them, using heavy nitrogen as marker, proved this suggestion to be true.

Lipid metabolism on the lines initiated by Schoenheimer and Rittenberg was further investigated in recent years by their former associates and others, such as Bernhard (*cf.* 1943, 1944, 1945), Keston (*cf.* 1942, 1944), Sperry, Stetten, Stoyanoff, Waelsch (*cf.* Waelsch *et al.*, 1939, 1941a and b, 1942) and others. Cavanagh and Raper (1936, 1939) studied fat metabolism at an early date, using deuterium as an indicator. The proof that methionine is a precursor of choline insofar as the methyl groups are concerned was brought by du Vigneaud and associates (1941a), while Stetten (*cf.* 1941, 1942a and b, 1943, 1945, 1946a and b) and also Ussing (1938) concentrated their efforts on

the elucidation of the problems of carbohydrate metabolism.

Tritium has so far found a restricted application as a tracer, which is mainly due to the formerly great difficulties of obtaining this isotope. Allen and Ruben (1942) have shown by studying the oxidation of fumaric acid with permanganate in tritium water that the C — H bond is not labilized during oxidation. Furthermore, Norris *et al.* (1942) applied tritium in the study of how far chlorophyll participates as a hydrogen donor in the manner analogous to the action of coenzymes in the respiratory cycle.

The Schoenheimer group (Schoenheimer *et al.*, 1939) and, simultaneously, Ussing (1939) introduced an important method of quantitative biochemical analysis, that of "isotope dilution," in which deuterium or nitrogen (Rittenberg and Foster, 1940) labeled amino acids and other labeled compounds were applied.

Heavy Nitrogen as a Tracer

To Urey we owe not only the discovery of the important isotopic marker deuterium, but also of methods for the concentration of stable isotopes, such as C^{13}, N^{15} and O^{18}. After having discovered the fact that the animal body fats are in a state of rapid flux, and having elucidated numerous steps of their metabolism, Schoenheimer and Rittenberg (1939), making use of the above mentioned great advance, focused their attention on an analogous study of protein metabolism. Amino acids synthesized from isotopic ammonia and added in small amounts to the diet of adult rats in nitrogen equilibrium were found to be rapidly and extensively incorporated into tissue proteins. After the ingestion of isotopic amino acids or ammonia, heavy nitrogen was found in all amino acids isolated from the proteins, except lysine. According to the concept of independent exogenous and endogenous metabolism, most of the dietary nitrogen should have appeared directly in the urine. With leucine, for example, less than one-third was excreted.

In some investigations, doubly marked amino acids were employed, as, for example, leucine labeled both with N^{15} and H^2. The samples of leucine obtained from the bodies of the leucine-fed animals contained a very high concentration of marked nitrogen, which indicated that the dietary leucine actually had replaced leucine in the protein. Moreover, as the isolated leucine contained deuterium, not only the amino group, but the whole molecule was introduced. When doubly marked lysine was fed, a very different result was obtained. The concentra- of both isotopes, N^{15} and H^2, in the lysine isolated was lower than those in the compound administered (owing to the admixture with normal, non-isotopic lysine), but the ratio of the isotope concentrations remained the same. The results show that, in contradistinction to other amino acids studied, none of the nitrogen of the α-amino group of lysine had been replaced by nitrogen from other sources (Weissman and Schoenheimer, 1941). Simultaneous application of N^{15} and H^2 as markers led to many other important results, such as those obtained in the studies of du Vigneaud *et al.* (1939) on the biological inversion of amino acids of "unnatural" steric configuration. They proved also (1946c) the ability of sarcosine to serve as a methyl donator for creatine and choline (*cf.* also 1940, 1941).

Several investigations of the Schoenheimer group deal with the study of structural elements in the formation of excretory products. The main nitrogenous constituents of the urine are urea, ammonia, creatine and uric acid. The formation of these substances is closely interrelated with those processes to which the nitrogenous tissue components are subjected. These investigations lead, among others, to the complete elucidation of the precursors of creatine, which were found to be glycine, arginine and methionine. The glycine and arginine were found by Bloch and Schoenheimer (1941) to be precursors of the α-amino acid and guanido groups. A paper of outstanding importance was published by Bloch, Schoenheimer and Rittenberg in 1941, in which the rate of creatine formation in normal rats on a creatine-free diet was investigated with the aid of two different isotopic creatine preparations, one containing N^{15} in the sarcosine part only and one containing N^{15} in the amidine as well as in the sarcosine part of the molecule. Within 29 days, half of the creatine molecules in adult rats on a creatine-free diet were found to be replaced by new molecules. It was also found that, in contrast to the amidine moiety of arginine, that of creatine is not replaced during metabolism. Recently, the rate of turnover of endogenous creatine on a diet as free of creatine as of creatinine was found to be 1.6 percent per day (Hoberman *et al.*, 1948).

After investigating to what extent ammonia is an intermediate in the transfer of amino groups from one amino acid to others and of the process of transamination, the Schoenheimer group turned its attention to the problem of the presence of reserve proteins in the animal body. This question could be tested by isolating from the experimental animals such proteins as may be considered to be specific. Such a plan was hampered by the scarcity of heavy nitrogen at that time; recourse was therefore taken to immunological procedures (Schoenheimer *et al.*, 1942; Heidelberger *et al.*, 1942).

The amino acid replacement and nitrogen transfer among individual amino acids was found to occur in antibody and normal serum proteins, in the same manner as has been indicated in organ protein. The average rate of these reactions has been observed in antibody and normal serum proteins by following the rate of replacement of isotopic nitrogen by normal nitrogen. The half-life of an antibody molecule was found to be about two weeks, approximately the same as that of the average serum mole-

cule. The half-life time of liver proteins was determined by Shemin and Rittenberg (1944) to be seven days.

A very different behavior was shown by the passively injected antibody, which does not undergo changes involving nitrogen replacement. As the possibility of continued antibody formation is lacking, no synthesis of antibody protein involving incorporation of marked nitrogen takes place.

Barnes and Schoenheimer (1943) and Plentl and Schoenheimer (1944) have applied heavy nitrogen in the study of nucleic acid metabolism. They found that none of the purines or pyrimidines tested in the diet were utilized by the body for the synthesis of nucleoproteins. Recently, Brown and associates (1947) found that adenine present in the diet, in contrast to guanidine, is utilized for the synthesis of nucleic acids. They have shown also that adenine, labeled in the pyramidine nitrogens, is converted to guanine with labeled nitrogen still in the same positions in the purine ring. Sonne *et al.* (1946) and Shemin and Rittenberg (1947) have recently shown that glycine is utilized for uric acid synthesis in both pigeon and man, and Abrams *et al.* (1948) have brought evidence that glycine plays the same role in the synthesis of purines in yeast. The work of Kalckar and Rittenberg (1947) brought information about the rate of rejuvenation of the 6-amino group of adenosine triphosphate from muscle, which was found to be a rapid process in contrast to the rate of rejuvenation of the ring N.

The investigations described in the study of nucleic acid metabolism were the last ones in which the great pioneer participated. The line of research initiated by him and Rittenberg was, after Schoenheimer's untimely death, continued with further great success in his former department and in other institutions. It is hardly possible within the scope of this report to enumerate all the papers published. I want to mention only Shemin and Rittenberg's (1946a and b) study of the biological utilization of glycine for the synthesis of protoporphyrin, of hemoglobin, and the application of labeled heme in the determination of the life-span of human red corpuscles. They also investigated the origin of glycine. Glycine was shown to be a nitrogenous precursor of the protophorphyrin of hemoglobin in the rat. Study of the isotope concentration found in the heme of the human red corpuscles, after feeding of glycine labeled with N^{15}, led to the result that the life-span of human red cells is 127 days.

In his studies of the origin of glycine in the rat, Shemin (1946) found the formation of glycine from serine to occur even though the diet contained an adequate supply of glycine, giving another example of the fact generally observed in tracer work that, if the living cell has a mechanism for the formation of a certain compound, the synthesis occurs regardless of the amount of the compound present in the diet. As to the mechanism of the formation of glycine from serine, he found in an investigation in which serine, labeled by N^{15} in the amino group and by C^{13} in the carboxyl carbon was fed, that the β carbon atom of serine is oxidized to an aldehyde, which, by a reverse aldolyzation, yields glycine. The conversion of the carbon skeleton into cystine was formerly shown by Stetten (1942a), who fed labeled serine to rats; he found also (1941, 1942b) by feeding labeled choline or ethanolamine that the conversion of ethanolamine into choline proceeds without hindrance in rats even when the diet is sufficiently poor in methyl groups to cause fatty liver. It was furthermore demonstrated by du Vigneaud and associates (1946b) that methyl groups from dietary betaine appear in tissue choline almost as rapidly as they appear from dietary deuteriocholine. In these investigations, betaine labeled with deuteriomethyl groups and N^{15} was synthesized and fed to growing rats. The studies were extended to the relationship of monomethyl- and dimethylaminoethanol to choline and transmethylation reactions (du Vigneaud *et al.*, 1946a).

Application of heavy nitrogen in botanical studies

Not only in the animal organism but also in plants, N^{15} was found to be incorporated into the protein molecules, even if little growth occurred. Vickery and associates (1939, 1940) found in a buckwheat plant of 29 days of age, that in the course of two days six percent of the protein nitrogen had undergone replacement by interaction with the simple nitrogenous substances of the cells, among which were ammonia and amide nitrogen of augmented isotope ratio. Also in experiments with sunflowers placed in a culture solution containing heavy ammonium nitrate a renewal of the protein molecules present in the different parts of the plant was demonstrated (Hevesy and associates, 1940). N^{15} supplied to *Azotobacter vinelandii* accumulates in the glutamic fraction (Burris, 1942) which contains the highest level of N^{15}. Recent work of Wilson and Burris (1947) points to NH_3 as the key intermediate for Azotobacter. Amino acids show a distribution very similar to that observed when N^{15} was supplied as molecular nitrogen (Burris and Wilson, 1946). The short lived radionitrogen was applied in the study of nitrogen fixation by non-leguminous plants by Ruben *et al.* (1940a).

Heavy Oxygen as a Tracer

Heavy oxygen has found so far a restricted application in tracer work, which is due partly to the great difficulty of obtaining this isotope. Though oxygen cannot compete with nitrogen as to importance in indicator work, it is a useful tracer and will presumably be used on a larger scale now that it has become more easily available. The main application of O^{18} lies so far in the field of elucidation of reaction mechanisms. Polányi and Szabó (1934) first used heavy oxygen in this type of study, eluci-

dating the mechanism of the alkaline hydrolysis of amylacetate. In a similar way, Bentley (1948) recently studied the mechanism of hydrolysis of acetylphosphate.

The fate of the sulfate radical in the animal body was investigated by Aten and Hevesy (1938). When the photosynthetic process of *Chlorella pyrenoidosa* was carried out in water enriched with respect to the O^{18} isotope, the $O^{18}:O^{16}$ ratio of the evolved oxygen was equal to that of the water used. When the algae were grown in ordinary water containing enriched $K_2CO_3^{18}$ and $KHCO_3^{18}$, the isotope ratio of the evolved oxygen did not show any enrichment (Ruben *et al.*, 1941).

Application of Artificially Produced Radioisotopes

During our early work with "natural" radioisotopes as indicators, we often mentioned what an attractive place that Fairyland must be where radioactive indicators of all elements are available. This utopia became reality almost with a single stroke, when the Joliot-Curies made their most important discovery of artificial radioactivity. The path was thus paved for investigation of the fate of the atoms of the common constituents of the animal and plant organisms. The fact that we gave priority to radiophosphorus in our early studies with the late Dr. Chievitz, in which we were assisted by Dr. Hilde Levi, was due partly to the convenient way in which radiophosphorus can be produced and its radiation measured, and partly to the great significance of phosphorylation processes taking place in the organism, phosphorus compounds being involved in carbohydrate and fat metabolism, in skeleton formation, in mitotic processes and so on. We started our researches before the advent of the cyclotron, having at our disposal a mixture of radium emanation and beryllium, and later of radium sulfate (600 millicuries) and beryllium. The neutrons emitted by such a radium source were to a large extent absorbed by 10 l. of CS_2. By shaking the irradiated CS_2 with dilute nitric acid or with water only a few microcuries of P^{32} could easily and conveniently be obtained. When such labeled sodium phosphate of negligible weight was administered to rats, within a few hours the presence of an appreciable amount of P^{32} in all organs could be ascertained. As the P^{32} administered must be expected to show the same behavior as the P^{31} present as inorganic phosphate in the circulating plasma, these early results (Chievitz and Hevesy, 1935, 1937) demonstrate that a replacement of the phosphorus content of the organs, or at least of a part of the phosphorus atoms present in the organs, by phosphorus atoms of the plasma and *vice versa*, takes place incessantly. The dynamic nature of the phosphorus compounds present in the animal organism was thus demonstrated. A similar result was thus obtained at about the same time by Schoenheimer and Rittenberg in their classical investigations previously described. I want first to describe the application of radioactive tracers in the study of skelton formation, a problem first attacked in the above mentioned experiments.

Radioactive Tracers in Skeleton Formation

In early investigations in Copenhagen (Hevesy *et al.*, 1937a) the uptake of P^{32} by the growing incisors of the rat was compared with the P^{32} uptake by non-growing bones and teeth of the adult rat. The P^{32} uptake by the enamel of equal weight was found to be at least 20 times as small as that found in the molars of the mature rat. The difficulty encountered in those days in such types of investigations are demonstrated by the fact that, while 26 counts per minute were produced by a dentine sample weighing 227 mg., one enamel sample (33.1 mg.) separated by grinding it off from the dentine showed 0.6 counts per minute, only; another one (19.1 mg.) separated from the uppermost enamel layer showed 0.7 counts. Minute activities had thus been measured. The finding that the uppermost enamel layer shows a greater P^{32} content than the more deeply located enamel layer was found to be correct by numerous later investigations, summarized by Armstrong (1942; *cf.* Sognnaes and Volker, 1941; Pedersen *et al.*, 1942; Bevelander and Almer, 1945), in which it was also proved that the higher activity of the uppermost enamel layer is due to an uptake of P^{32} by the enamel from the saliva and that this can be avoided by protecting the tooth by a cap.

The work of Dols and associates (1938, 1939) belongs to the earliest skeletal studies making use of P^{32}. These authors compared the uptake of P^{32} by the bones of normal and rachitic rats and chickens, respectively. As shown in these and other investigations, the epiphysial bones take up more P^{32} than the diaphysial ones, these investigations demonstrating the pronouncedly epiphysial character of the rachitic skeleton.

When rats were sacrificed at different dates after administration of the same dose of P^{32}, the radiophosphorus content of the skeleton was found, after the lapse of 98 days, to comprise 92 percent of the total P^{32} content of the rat, which compares with only 18 percent found after the lapse of one-half hour (Hevesy, 1939). These and similar results (Manly and Bale, 1939; Manly *et al.*, 1940) demonstrate that, while a part of the skeleton phosphorus interchanges easily with plasma or lymph phosphorus, a large part of the bone phosphorus is renewed at a very slow rate only. In view of the continuous decline of the P^{32} content of the plasma inorganic P great difficulties are encountered in calculating, from plasma and skeleton data, to what extent the skeleton P was renewed during the experiment. Difficuties in the interpretation of the results, due to the change of the activity of the phosphorus fractions during the experiment, were discussed in an early paper by Artom and associates (1938), who

carried out calculations designed to help eliminate these difficulties. The incorporation of P^{32} in the skeleton follows such intricate lines that one encounters the greatest difficulties in calculating renewal figures from activity data in experiments in which the P^{32} dose is administered at the start of the experiment. Different newly formed molecular layers of the bone apatite crystals will show a very different activity, corresponding to the (unknown) data at which they are found. Where the incorporation of P^{32} in the bones follows such intricate lines, mathematical considerations will hardly help us to evaluate renewal figures from plasma activity and bone activity values ascertained at the end of the experiment. These difficulties can be eliminated by administering P^{32} to the animal repeatedly throughout the experiment and thereby keeping its plasma activity at a constant level. Under such conditions the newly formed apatite layers will show the same specific activity (activity per mg. P). Such experiments (Hevesy *et al.*, 1940) brought out the fact that 74 percent of the epiphysial and 93 percent of the diaphysial parts of the tibia or the femur of the adult rabbit remain unchanged in the course of 50 davs (*cf.* also Armstrong, 1947).

While corresponding experiments with radiocalcium are still to be performed, Armstrong and Barnum (1947) applied P^{32} and Ca^{45} simultaneously in their studies and compared the rate at which these two isotopes are taken up by the skeleton in the later phases. Some differences were found.

The effect of vitamin D on deposition of phosphorus or calcium can be conveniently investigated by using radioactive tracers (Morgareidge and Manly, 1939; Shimotori and Morgan, 1943). As to the uptake of calcium, Greenberg (1945), applying Ca^{45} and Sr^{89} could show that vitamin D favors the absorption of calcium from the intestinal tract and directly influences the mineralization of the bone as well. The increased absorption under the effect of vitamin D was also demonstrated by using Sr^{45} (Weissberger and Harris, 1943). Extended studies were also carried out on the absorption of P^{32}, Na^{24} and so on, by powdered bone (Manly and Levy, 1939; Armstrong, 1940; Hodge and Falkenheim, 1945; Falkenheim *et al.*, 1947).

Using C^{11}, Solomon and co-workers (1941) after the lapse of 2.5 hours found 1.8 percent of the labeled carbonate administered to rats to be present in the bone carbonate. Radioautographs of bones of rats injected with C^{14} show a markedly different picture (Bloom *et al.*, 1947) from those of bones after injection of P^{32} and Sr^{89}. The C^{14} injected as carbonate appears primarily in those areas occupied by preexisting bone. Fission products and the heaviest elements, as indicated by the radioautographs taken by Hamilton and associates (Hamilton, 1947; Copp *et al.*, 1947) are also deposited by some mechanism in the bone tissue other than that of the alkaline earth metals.

Autoradiographs in distribution studies were first introduced by Lacassagne and Lattes (1924), who investigated the distribution of polonium in the animal organism. Lomholt (1930) followed the distribution of labeled lead, taking autoradiographs. Dols *et al.* (1939) compared the uptake of P^{32} by normal and rachitic bones. Hamilton and associates (1940) made extensive use of autoradiographs in their studies on the uptake of iodine by the thyroid gland. Arnon *et al.* (1940) studied the uptake of phosphorus by tomato fruits at various stages of development. Autoradiographs were used in the identification of the iodine storing tissue in an Ascidian (Gorbman, 1941) and in numerous other cases of iodine storage, and may become very useful in localizing tracer substances in different regions of tissue cells. Autoradiographs were also found a useful tool in the studies of Axelrod and Hamilton (1947) on the distribution of mustard gas in the skin and eye tissues. In recent years, the technique of autoradiographs has been much improved (Leblond *et al.*, 1946). Though counting of the number of emitted particles and electrometric measurement are mainly used in tracer technique, the photographic method proved to be useful in studies like the uptake of radioactive isotopes by the bone tissue, the uptake of radioiodine by the thyroid gland, and so on.

Phosphorus Turnover in Soft Tissues

Turnover of acid-soluble phosphorus compounds

Early investigations with radiophosphorus (Hevesy, 1938, 1939) brought out great differences in the rate of penetration of phosphate into the cells of different organs. Phosphate was found to penetrate into the liver cells at a remarkable rate, while the permeability of muscle cells and also of the red corpuscles was found to be low. However, after the phosphate ions reached the cells, they were found to participate with a remarkable rapidity in the phosphorylation processes taking place in the cells; thus labeled pyrophosphate, creatinephosphoric acid, and many other acid-soluble phosphorus compounds are formed at a very rapid rate. This process, slow penetration followed by rapid turnover of many acid-soluble phosphorus compounds, is most easily observed when studying the phosphorus interchange between plasma and red corpuscles (Hevesy and Aten, 1939; Hevesy and Hahn, 1940a). That the specific activity of several organic P fractions lags behind the specific activity of the plasma inorganic P is due to a slow penetration of the tracer into the cells, and not to a low rate of renewal of the organic compounds.

An analogous behavior is shown by tissue cells where, however, in experiments of shorter duration due regard must be taken to the fact that the inorganic P secured from the tissues is partly of extracellular origin. By assuming the extracellular inorganic P to have the same specific activity as the

plasma inorganic P, and the percentage content of inorganic P of the plasma and the interspace fluid to be equal, we can account for the share of the activity of the extracellular P in the tissue inorganic P, and thus calculate the renewal figures (Hevesy and Hahn, 1940b). An effective way to eliminate the extracellular P is, as shown by Kalckar *et al.* (1943), to perform viviperfusion. In experiments with surviving muscle slices in which the extracellular P was removed by prolonged washing (Furchgott and Shorr, 1943), the extent of renewal of creatine phosphate and of the labile phosphorus of ATP molecules was found, even at 2° C, to be appreciable, a similar result being obtained with frogs kept at 2° C (Hevesy *et al.*, 1941). Using myosine and adenosine triphosphatase as a tool for differentiating between the two labile group of ATP, Flock and Bollman (1943, 1944) found, in experiments lasting one hour, a higher P^{32} concentration in the terminal phosphate group than in the second phosphate group. The same result was obtained by Furchgott and Shorr (1943). That the third P atom of ATP is renewed at a much slower rate than pyrophosphate P was shown at an early date by Korzybski and Parnas (1939).

Sacks (1940, 1944, 1945; *cf.* also Sacks and Altschuler, 1943) carried out extensive studies of the uptake of P^{32} by acid-soluble phosphorus compounds, and of the effect of stimulation, recovery, glucose and insulin administration on phosphorus turnover in muscles; while Kaplan and Greenberg (1943, 1944) studied the turnover of acid-soluble phosphorus compounds in the liver. In experiments on perfused cat liver at an early date, a high percentage of the ATP molecules and a minor percentage of the ester phosphorus were found by Lundsgaard (1938) to be renewed in the course of one hour.

Radiophosphorus was used by Borell and coworkers (1947) in the study of metabolic processes taking place in the tuber cinereum, adenohypophysis, and ovaries in the different phases of the sexual cycle of the rabbit.

Experiments *in vitro,* in which much simpler conditions prevail than *in vivo,* were used at an early date in the studies of various phases of phosphorylation processes carried out by a combined group of Copenhagen and Warsaw scientists (Hevesy *et al.*, 1938; Parnas, 1938, 1939) and by Meyerhoff and his associates (1938), respectively. Studying the transformation of glucose-1-phosphoric acid into glucose-6-phosphoric acid in the presence of labeled inorganic phosphate, the esters were found not to become active.

Meyerhoff *et al.* (1938) determined by *in vitro* experiments the rate of renewal of the labile P in ATP. The average time of interchange of a pyrophosphate group with a phosphate group was shown to be only fifty seconds. By using radioactive phosphate, the fairly rapid exchange between inorganic and acetyl-bound phosphate in extracts of *Clostridium butylicum* and of *Escherichia coli* was studied by Lipmann and Tuttle (1945). In this connection, experiments by Chargaff and co-workers (1945) may be mentioned. These authors, making use of radioactive thromboplastin, showed that the amount of phosphorus-containing compounds transferred to the thrombin, if at all significant, is extremely small.

Turnover of Phosphatides

Artom and associates published in 1937 and 1938 the results of their important investigations on the turnover of phosphatides in the animal organism. This was also the subject of numerous extended investigations of the Chaikoff group (Perlman *et al.*, 1937; Entenman *et al.*, 1938; Fries *et al.*, 1938, 1942; Schachner *et al.*, 1942). Taurog and associates (1942) demonstrated that respiratory inhibitors exclude the formation of labeled phosphatides in tissue slices.

The elucidation of the effect of lipotropic substances on phosphatide formation is the subject of numerous investigations carried on by Chaikoff and his colleagues (Perlman and Chaikoff, 1939; Chaikoff, 1942; Friedlander *et al.*, 1945; Entenman *et al.*, 1946). The same topic was investigated by Patterson and co-workers (1944) and, recently, by Platt and Porter (1947).

In connection with the early experiments mentioned above, Copenhagen workers investigated (Hahn and Hevesy, 1937) whether, in spite of the absence of a new formation of cells in the brain of the adult animal, a renewal of phosphatide molecules can be observed. A slow but clearly indicated renewal was found to take place. A detailed study of the formation of labeled phosphatides in the brain of rats of very different ages was carried out by Changus *et al.* (1938) and by Fries and co-workers (1940).

Attempts were also made at an early date to apply radioactive indicators in the study of the origin of the phosphatides present in the yolk (Hevesy and Hahn, 1938) and in the milk (Aten and Hevesy, 1938*b*; Aten, 1939). Labeled phosphatides of the plasma were shown to be incorporated into the yolk during its formation in the ovary. The activity level of plasma phosphatides was found, in experiments lasting a few hours, to be higher than that of the phosphatides of any organ except that of the liver, indicating the liver to be the main source of the plasma and yolk phosphatides. The labeled phosphatide molecules present in the milk of the goat were found to a large extent at least to be built up in the mammary gland. Entenman and associates (1938) compared the uptake of P^{32} by various organs of laying and nonlaying hens, while Lorenz *et al.* (1942) followed the incorporation of administered P^{32} in the different parts of the egg. Chargaff (1942a) found labeled vitelin to be formed in the yolk at a more rapid rate than labeled phosphatides. That not only labeled phosphatides are formed in

the liver at a more rapid rate than in any other organ, but that the phosphatide molecules present in the liver penetrate easily into the plasma and *vice versa,* was shown in experiments in which plasma containing labeled phosphatides was injected into the circulation of the rabbit (Hevesy and Hahn, 1940c) or the dog (Zilversmit *et al.* 1943 a and b). In the course of a few hours, an appreciable part of the labeled phosphatides was found to be located in various organs and mainly in the liver. The Chaikoff group extended their investigations to liverless dogs. Labeled phosphatides were found to disappear from the plasma of liverless dogs six to ten times more slowly than from the circulation of normal dogs (Entenman *et al.,* 1946), bringing thus further important evidence that the plasma phosphatides derive mainly from the liver. No less striking evidence is brought by other results of the above mentioned group. When labeled phosphate was administered to a hepatectomized dog, the amounts of labeled phosphatides formed in the kidneys or the intestinal mucosa did not differ from that found in the intact dog, nevertheless only negligible amounts of phosphatide P^{32} were recovered from the plasma of the hepatectomized dog (Fishler *et al.,* 1943). Among numerous other investigations of the Chaikoff group, that of Reinhardt *et al.* (1944) should be mentioned, in which a part of the labeled phosphatide molecules injected into the plasma reached lymphatic channels and were recovered in the thoracic duct lymph. Chargaff (1939) was the first to show that a restricted difference is found only in the rate of formation of lecithin and cephalin in various organs. This was also shown by, among others, Hahn and Thyrén (1945).

The results obtained in the above mentioned investigations are based on the comparison of turnover rates expressed in arbitrary ("relative") units. The solution of some other problems may necessitate the knowledge of absolute turnover figures. That is the case, for example, if we have to decide whether phosphatide formation is an obligatory step in fat oxidation or transfer, or not, a question to which a negative answer is given by Flock and Bollman (1945). The ratio of specific activities of phosphatide P at the end of the experiment and the average cellular inorganic P during the experiment of short duration multiplied by 100 gives the percentage turnover rate (Hevesy and Hahn, 1940b). In experiments of longer duration the repeated renewal of phosphatide molecules must be taken into account as well. This calculation is based on the assumption that the precursor of phosphatide P is cellular inorganic P or organic P which gets into rapid exchange equilibrium with the cellular inorganic P. A detailed study of the relation between the specific activity of a compound and its precursor was made by Zilversmit *et al.* (1943b). It is a problem of great importance how far all phosphatide or other molecules present in an organ have the same chance of being renewed. When investigating phosphatide turnover in the cell nuclei and the cytoplasm of the liver (Hevesy, 1946), pronounced differences were found. Furthermore, Kamen *et al.* (1947) isolated from yeast two metaphosphate fractions of very different specific activity.

Turnover of nucleic acids

Early investigations (Hahn and Hevesy, 1940) have shown that, in contrast to other phosphorus compounds, desoxyribose nucleic acid has a low rate of renewal in the liver, a high figure being found for the turnover taking place in the thymus gland. Ribosenucleic acid was found to be turned over at a more rapid rate in the liver than desoxyribosenucleic acid, and for both much higher figures were obtained in the regenerating than in the resting liver (Brues *et al.,* 1942, 1944). Nucleic acids are turned over more rapidly in cancerous than in normal liver (Kohman and Rusch, 1941). The total ribosenucleic acid present in the rat is renewed at a rate about twice as rapid as the total desoxyribosenucleic acid (Hammarsten and Hevesy, 1946). The rate of formation of labeled nucleic acid is appreciably reduced by irradiation with Roentgen rays (Euler and Hevesy, 1942, 1944; Ahlström *et al.,* 1945, 1946; Jones, 1946). Marshak (1947) investigated recently the effect of desoxyribosenuclease on labeled cell nuclei, which was found to split off P of much lower specific activity than obtained after treatment of the cell nuclei with ribosenuclease. Among the more recent observations on nucleic acid turnover, Spiegelman and Kamen (1946) found the turnover of ribosenucleic acid of yeast in the absence of nitrogen to be quite low.

Uptake and excretion of P^{32} by growing tissue

The uptake and excretion of the total labeled phosphorus by the outgrown and growing tissues was in many cases investigated in connection with the medical application of P^{32}. Among these are numerous investigations of Lawrence and coworkers (Lawrence and Scott, 1939; Lawrence *et al.,* 1939; Erf *et al.,* 1940, 1941; Lawrence *et al.,* 1940; Erf and Lawrence, 1941; Tuttle *et al.,* 1941), one of the first group of investigations of this type being those of Scott and Cook (1937), Cohn and Greenberg (1938), and Hahn *et al.* (1938). Tissue grown in labeled medium is bound to contain labeled compounds. Growing tissue contains more P^{32} than fully grown tissue, as brought out by the above mentioned and other investigations. Resting ovary of rabbits weighing 0.16 g. takes up only five units of administered P^{32} (Bulliard *et al.,* 1939), while the corpus luteum weighing 0.25 g. in the same experiment takes up 100 units. The findings of Lawrence and associates (1939) that the proportion of P^{32} per gram in various tissues of the leukemic mouse is greater than that in the normal mouse, and corresponding observations made by Warren (1943) on human material, present

other examples of enhanced incorporation of P^{32} in growing and rapidly metabolizing tissue. The ratio between the radioactivity measured per kilogram of tissue and the amount of P^{32} administered per kilogram of body weight is denoted by Kenney *et al.* (1941) as "differential absorption ratio," and determined by them and Forssberg and Jacobsen (1945) for numerous organs, tumors and metastases. Tumors and metastases concentrate P^{32} to a higher extent than do the organs investigated, with the exception however of such active organs as the liver, for example. Nuclei of rapidly multiplying cells (Marshak, 1940, 1941) take up more P^{32} than those of normal cells.

Many of the above quoted investigations contain data on the excretion of P^{32}. Hevesy *et al.* (1939), Kjerulf-Jensen (1941), and Gowaerts and Lambrechts (1942) determined to what extent the feces P is of exogenous and endogenous origin, respectively. From the numerous other studies in which labeled phosphate has found application, an investigation by Hahn and co-workers (1945) should be mentioned here; a physiological bilaterality of blood flow in the portal vein, which is streamlined in nature, was found.

Application of P^{32} in botanical studies

The replacement of a large part of the phosphorus atoms present in various compounds in the different parts of corn and sunflower seedlings by phosphorus atoms from the nutritive solution was demonstrated by making use of P^{32} as a tracer (Hevesy and associates 1936, 1937b). The speed with which labeled phosphorus contained in a culture solution invades bean seedlings was measured, and P^{32} was found to migrate 10 cm. per hour (Biddulph, 1939, 1941). The migration of P^{32} in corn and wheat seedlings was also followed by Brewer and Bramley (1940). Arnon *et al.* (1940) applied P^{32} to the study of phosphorus absorption by tomato fruits at various stages of development. By applying labeled phosphorus, sodium and potassium, respectively, Stout and Hoagland (1939) brought evidence that the xylem and not the phloem is the path of rapid upward movement of salts. The rate of absorption of phosphorus by soils and its movement through the soil was studied by Ballard and Dean (1941).

Application of Radiosulfur

Prior to the availability of pile radiosulfur, S^{35} was much more difficult to obtain than P^{32}. This and the less convenient measurability of the sulfur radiation are, however, not the only reasons why more tracer investigations were carried out with radiophosphorus than with radiosulfur. In contrast to numerous organic phosphorus compounds of great biochemical importance, the number of organic sulfur compounds present in animal and plant organisms is more restricted. The animal organism cannot avail itself of the sulfate group in the formation of these compounds, as shown by early experiments of Tarver and Schmidt (1939) using tagged sulfur. In spite of these facts, a number of important investigations were carried out in which radiosulfur was applied as indicator. Most of these investigations on sulfur turnover were carried out by Tarver and his co-workers. These studies include, among other things, an investigation of the rate of replacement of protein sulfur by labeled sulfur in the animal body (Tarver and Schmidt, 1942; Tarver and Reinhardt, 1947). Besides S^{35}, the stable isotope S^{34} was also applied in the study of conversion of methionine to cystine (du Vigneaud and associates, 1944).

When surviving liver slices of the rat were incubated in the presence of labeled methionine, about one percent of the labeled substrate was found to be taken up per gram of liver slice (Melchior and Tarver, 1947). Simmonds, Cohn and du Vigneaud (1947) fed young dehaired rats methionine containing an excess of S^{34} and with the β and μ carbons labeled with C^{13}; 80 percent of the sulfur of the cystine isolated from the new coat of hair was derived from the dietary methionine, but no significant amount of the carbon of the cystine was isotopic. Cystathionine was found to be an intermediate of cystine formation.

Recently (Boursnell *et al.*, 1947), S^{35} and also P^{32} found application in immunological investigations.

Proteins were tagged with S^{35} and their disappearance studied by Seligman and Fine (1943). Radiosulfur found application, among others, in the study of sulfur fed as sodium sulfide to rats (Dziewiatkowski, 1945, 1946), and in the study of thiamin metabolism in man (Borsook *et al.*, 1941), while the Wormall group carried out very extended studies of the fate of mustard gas, the sulfone and the sulfoxide in the animal body (Boursnell *et al.*, 1946; Banks *et al.*, 1946).

Radioselenium was applied by McConnell (1942) in time-excretion studies of exhaled selenium. In contrast to selenium, very little tellurium is excreted through the respiratory tract (De Meio *et al.*, 1947).

From among the botanical applications of radiosulfur, the work of Thomas *et alia* (1944) should be mentioned. High initial accumulation of sulfur was observed in the leaves followed by a steady lowering as it was redistributed.

Application of Radioactive Isotopes of Extracellular Elements

Radioactive isotopes of elements mainly present in the extracellular fluid, as chlorine, bromine and sodium, were in numerous cases applied in permeability measurements, etc. Griffiths and Maegraith (1939), Manery and Bale (1939), Hahn *et al.*, (1939), Greenberg *et al.* (1940), Kaltreiter *et al.* (1940, 1941) applied labeled sodium, while Manery and Haege (1941) applied labeled chloride in the determination of the extracellular space. Hahn and

Hevesy (1941) studied the rate of disappearance of these and other ions from the circulation of the rabbit following intravenous injection and carried out extended studies of the extracellular volume. Determinations were carried out of the permeability of the placenta by the Flexner group (Flexner and Pohl, 1940, 1941; Gellhorn *et al.*, 1943; Wilde *et al.*, 1946, and numerous other papers), while the permeability of blood-cerebrospinal fluid barrier was studied by Greenberg and associates (1943) and the rate of penetration into the aqueous humor by Barany (1946). The movement of sodium ions between the intestinal lumen and blood was also studied by Visscher and coworkers (1944). The rate of resorption and absorption of the above mentioned and other ions from the intestinal tract was studied by Hamilton (1937, 1938) and by Hamilton and Stone (1937) in early investigations. Ussing and associates (1947) studied the influence of the neurohypophyseal principles on the sodium metabolism in the axolotl.

Labeled sodium found an extended application in the determination of the circulation time in normal human subjects and subjects having peripheral vascular diseases (Hubbard *et al.*, 1942; Smith and Quimby, 1944, 1945; Quimby, 1947; Reaser and co-workers, 1946; Thompson and associates, 1946). Measurements of the circulation time were previously carried out with natural radioactive bodies by Blumgart and Weiss (1927). Fox and Keston (1945) applied Na^{24} in the study of the mechanism of shock and Cope *et al.* (1943) in the study of absorption of sodium from the stomach.

Investigations by Perlman *et al.*, (1941) brought out the fact that a substantial amount of administered bromide is taken up by the thyroid gland. Among numerous investigations in which the above mentioned radioactive isotopes found application, the study of the distribution of gaseous radiochlorine inhaled by mice (Born and Timoféef-Ressovsky, 1940), and the application of organic compounds containing radiobromine in the study of the permeability of capillaries to colloids (Cope and Moore, 1944) and in the diagnosis of localized inflammation (Moore and Tobin, 1942) should be mentioned. Daudel and associates (1946) studied the metabolism of labeled triphenyl ethylenbromide.

Radioiodine

When Hertz, Roberts and Evans (1938) and Hamilton (1938a) first reported physiological studies with radioiodine, the sole isotope then available was I^{128}, having a half-life time of only 26 minutes. Despite the short life of the tracer used, numerous interesting results were obtained. Hertz and associates found, among other things, that the normal rabbit thyroid could collect up to 80 times the quantity to be expected from uniform diffusion throughout the body tissue. In hyperplastic thyroids, indeed, this relative concentration could reach several hundredfold.

The discovery of I^{131}, having a half-life of eight days, very much facilitated the studies of iodine metabolism. Hamilton and Soley (1939, 1940) applied this tracer in extended studies of the uptake of iodine by human thyroids. In some of these experiments and in those by Hertz (1941), the uptake of iodine by the thyroids was measured by placing a Geiger counter tube against the gland. The radiographic technique found an extended application in some of the studies of Hamilton and associates (1940). Hertz and his colleagues (1940, 1941, 1942) extended their studies to iodine metabolism in Grave's disease, using I^{131}. The accumulation of iodine by metastases was investigated by Keston *et alia* (1942), that by certain types of thyroid carcinoma by Marinelli *et alia* (1947). The embryological development of the thyroid was demonstrated by radiographs of the tadpole of the frog (Gorbman and Evans, 1941).

The introduction of radioiodine as an indicator enlarged our knowledge of the relation of the thyrotropic hormone to iodine metabolism and thyroxine synthesis by the thyroid gland.

Mann *et alia* (1942) were the first to use labeled iodine to determine whether diiodothyrosine is the precursor of thyroxine. Much evidence is brought by the Chaikoff group in favor of the view that the formation of thyroxine is preceded by the formation of diiodothyrosine, both in experiments *in vivo* and in investigations of the uptake of iodine by surviving thyroid slices (Morton and Chaikoff, 1943).

Chaikoff and co-workers, after studying the uptake of labeled iodine by the thyroid gland (Perlman *et al.*, 1941b) carried out extended studies on the formation of protein-bound iodine in the plasma and on the role of anterior pituitary in iodine metabolism (Schachner *et al.*, 1944; Chaikoff and associates, 1947, and numerous other papers). The average rate of thyroxine secretion by the thyroid gland of the rat was determined by Taurog and Chaikoff (1947a) to be two γ per 100 grams of body weight per 24 hours. The fate of injected labeled thyroxine was moreover investigated by Joliot *et alia* (1944). Recently, further evidence has been presented by Taurog and Chaikoff (1947b) to show thyroxine to be the actual form in which the circulating thyroid hormone exists. Investigations carried out with radioiodine and other radioactive indicators by French scientists were recently surveyed by Daudel and Berger (1947).

The formation to a minor extent of diiodothyrosine and thyroxine of thyroidectomized animals was demonstrated by Morton *et alia* (1943). The activities of the Chaikoff group were extended, among other matters, to the study of the mechanism of action of various goitrogenic compounds. Much of this work was carried out by using thyroid slices (see Franklin *et al.*, 1944a and b; Keston *et al.*, 1944; Rawson *et al.*, 1944; Lawson *et al.*, 1945).

Astatine

Shortly after the discovery of astatine (eka-iodine) Hamilton and Soley (1940) compared the uptake of astatine and iodine by the thyroid gland. Astatine was found to accumulate in a manner similar to that of iodine.

Labeled Alkaline and Alkaline Earth Elements

The application of radium potassium by Brooks (1937, 1938) and later investigators (L. Hahn *et al.*, 1939; Eisenman and associates, 1940; Mullins *et al.*, 1941; Dean and associates, 1941; Cohn, 1941; Hevesy and Hahn, 1941; Levi, 1945) revealed the permeability of the red corpuscle membrane to potassium. Labeled sodium added to the plasma was found also to penetrate easily into the erythrocytes (Cohn, 1939; Hahn *et al.*, 1939; Eisenman and associates, 1940; Hahn and Hevesy 1942). The application of labeled ions thus revealed a new type of permeability which could be denoted as "interchange permeability" in contrast to the accumulation permeability measured by the usual chemical methods.

Extended investigations on the rate of interchange of plasma potassium with the potassium of the organs were carried out by Joseph *et al.* (1939) and especially by the Fenn group (Fenn *et al.*, 1941; Noonan *et al.*, 1941a). Muscular action (Noonan *et al.*, 1941b; Hahn and Hevesy, 1941) was found to promote markedly the interchange between muscle and plasma potassium. The entrance of Na^{24} into the muscle sodium of rats kept for a considerable time on potassium-free diet was observed by Heppel (1940). A rapid exchange of the sodium of the muscle fibres with that of the outside medium was found to occur (Ussing, 1947). The influence of neurohypophyseal principles on the sodium metabolism in the axolotl was recently shown by Barker Jörgensen and associates (1946) and by Ussing *et alia* (1947). Labeled sodium found application, among others in the study of the osmotic regulation in *Daphnia magna* by Holm-Jensen (1948).

In contradistinction to Sr^{89}, labeled calcium (Ca^{45}) only recently became accessible. The distribution of labeled strontium in the organs of the mouse was investigated by Pecher (1941) and compared with the uptake of calcium and phosphorus; that of calcium in the rat by Campbell and Greenberg (1940, *cf.* also Greenberg, 1945). An extensive uptake of Sr^{89} by the human skeleton was observed by Lawrence (1942).

Labeled Metals

By making use of Mn^{56}, of a mixture of Co^{56} and Co^{58} and of Fe^{55} as tracers, the excretion of manganese, cobalt, and iron by the dog was compared by Greenberg *et al.*, (1943). Studies on the distribution of manganese were also carried out by Born and associates (1943). In view of the importance of cobalt deficiency diseases in the livestock industry, detailed investigations were carried out on the excretion and tissue distribution of radiocobalt (Comar and Davis, 1947).

The entrance of copper into hematopoetic centers in amounts too small to permit detection by chemical methods has been demonstrated by Schultze and Simmons (1942), using radiocopper as a tracer. Other investigators (Yoshikawa *et al.*, 1942; Schubert *et al.*, 1943) determined the uptake of radiocopper by plasma and corpuscles.

Ely (1940) demonstrated the presence of radiogold in all tissues following the administration of gold. A more detailed investigation was carried out by Tobias and associates (1947).

Per gram of tissue, the pancreas was found most effective in taking up zinc, followed by liver and kidney (Sheline and co-workers, 1943).

The uptake of inert gases was studied by making use of radiokrypton by Cook and Sears (1945) and by Tobias and associates (1947). The incorporation of airborne fission products was studied by Scott and Hamilton (1948) and by Dalley *et alia* (1948).

Of all the metallic elements iron found the most extended application. The use of labeled iron in the study of the uptake of iron by the dog by Hahn and his colleagues (1939a and b, 1942) has shown that the body controls its iron stores by absorption or lack of absorption rather than by its capacity to eliminate iron. The results obtained made the presence of a ferritin-like substance in the intestinal mucosa appear highly probable (Balfour *et al.*, 1942). The very extended investigations of this group include, among others, studies of the utilization of absorbed iron in the body and its appearance in the red corpuscles (Miller and Hahn, 1940; Hahn and associates, 1942). In order to determine the amount of iron absorbed in diverse diseases, the percentage of administered radioiron present in the corpuscles of patients with several diseases was measured by Balfour and colleagues (1942). Hahn and associates (1943) studied the conversion of inorganic and hemoglobin iron into ferritin iron in the animal body. Several investigators observed newly administered iron to be used selectively in preference to body iron already stored (Dubach *et al.*, 1946; Ross, 1946; Greenberg and Wintrobe, 1946). Among the investigations dealing with the distribution of iron in the animal tissues those of Austoni and Greenberg (1940), Greenberg *et al.* (1943), Copp and Greenberg (1946), and Vanotti (1946) should be mentioned.

Application of Labeled Red Corpuscles

The dynamic structure of the living tissue was discovered by means of isotopic tracers. It is in some respects very fortunate that many of the enzymatic reactions take place in the animal and plant organism at a conveniently measurable rate. On the other hand, the incessant renewal of the tissue con-

stituents is very disturbing when one is faced with the task of labeling bacteria, virus or erythrocytes, for example, as the renewal of the molecules building up these systems may lead to a removal of the labeling agent. Radioiron and heavy nitrogen incorporated into the red corpuscles during their formation were, however, found to remain in the red corpuscles throughout their lifetime and found an extended application in labeling erythrocytes. In many cases, as in that of the determination of the circulating red corpuscle volume, a temporary labeling of the red corpuscles suffices equally well and, in these cases, P^{32} which in contrast to radioiron and heavy nitrogen can be introduced *in vitro* into the erythrocytes, found very extended application.

Hahn and associates (1940) found that, four hours after the administration of Fe^{59} to dogs made anemic by bleeding, some radioiron was present in the red corpuscles; and after a lapse of four to seven days practically all Fe^{59} absorbed iron was found in the erythrocytes. The incorporation of absorbed Fe^{59} into red corpuscles is so pronounced that Balfour and his colleagues (1942) used the Fe^{59} content of red corpuscles as a measure of the iron absorbed.

In the determination of circulating red corpuscle volume, a known volume of tagged corpuscles is introduced into the circulation. Blood samples are taken after a homogeneous mixture of injected and circulating blood is obtained. By comparing the activity of injected and secured corpuscle samples, the circulating blood volume is calculated. This principle was applied by L. Hahn and Hevesy (1940), Hevesy and Zerahn (1941) and others by using P^{32} labeled by P. F. Hahn and co-workers (1940, 1942), and others applying Fe^{59} labeled erythrocytes. Information was obtained on the passage of the red corpuscles through the peritoneum and the lymph spaces by using tagged red corpuscles (Gibson *et al.*, 1946a). The circulating red corpuscle volume in humans, using iron labeled erythrocytes, was determined in addition to the work mentioned above in numerous other studies (Ross and Chapin, 1942a, 1943; Gibson *et al.*, 1946b; Meneely *et al.*, 1947). The red corpuscle content determined by making use of labeled corpuscles is markedly lower than when estimated from venous hematocrit ratio and the plasma volume measured by blue dye dilution. The result suggests that the hematocrit of all circulating blood in the body is normally lower than that of blood drawn from large arteries and veins. Gibson and associates (1947b) succeeded in showing that the hematocrit of the blood in the large vessels is always less than that of arterial or venous blood. In this investigation, both with Fe^{55} and Fe^{59} tagged erythrocytes were applied Gibson and associates 1947a, b, c).

Besides their use in animal experiments mentioned on page 135, P^{32} labeled red corpuscles found numerous applications in the determination of the erythrocyte content of human subjects. Extended studies under very different conditions were carried out by Nylin and associates (Nylin and Malm, 1943, 1944; Nylin, 1945, 1947; Gernandt and Nylin 1946; Nylin and Hedlund, 1947; Nylin and Pannier, 1947; Nylin and Biörck, 1947). The red corpuscle content of human subjects was also determined by Hevesy and colleagues (1944). Naeslund and Nylin (1946) investigated the permeability of the placenta to red corpuscles.

While the red corpuscles conserve most of their P^{32} label for about an hour, thus far an interval which permits sufficient time to carry out determinations of the circulating red corpuscle volume, P^{32} red corpuscles cannot be applied in the determination of the life cycle of erythrocytes, nor can radioiron be applied in such determinations. In the case of nucleated red corpuscles alone was desoxyribonucleic acid P^{32} found to be a suitable indicator for the determination of the life cycle of the erythrocytes (Hevesy and Ottesen, 1945). The application of N^{15} labeled red corpuscles in the determination of the life cycle of mammalian erythrocytes was previously mentioned.

Labeled Carbon

Applications of C^{11} and C^{13}

Previously, only two isotopes of carbon had been available for biochemical studies, the stable isotope C^{13} and the radioactive isotope C^{11}. In recent years, C^{14} became available as well. It is a remarkable fact that numerous important results have been obtained by the use of C^{11} although its half-lifetime is only 20 minutes.

The use of C^{11} as a tool in biochemical research was first introduced by Ruben, Hassid and Kamen (1939) while C^{13} was first applied by Wood and associates (1940). In the course of their fundamental studies on photosynthesis, Ruben and co-workers (1940b) isolated from *Chlorella pyrenoidosa* exposed to an atmosphere of labeled CO_2 a radioactive fraction containing carboxyl and alcoholic groups. Plants which were allowed to grow in the light produced a photosynthetic intermediate which contained only a small fraction of the total C^{11} assimilated in the carboxyl groups; most of the activity resided in non-carboxyl atoms of the molecule. In contrast, when plants were exposed to $C^{11}O_2$ in the absence of light, a fraction was isolated which contained most of the radioactivity in the carboxyl groups (*cf.* Ruben *et al.*, 1940b; Overstreet *et al.*, 1940). Ruben and Kamen (1940b) found also that fresh yeast cells, suspended in water, assimilated CO_2. In their experiments, approximately one CO_2 molecule was reduced for every 50 molecules produced in respiration. Though most of the radioactivity was present in the precipitate formed by the addition of barium ions, the activity did not reside in the carboxyl groups of the barium precipitable material. Early experiments on photosynthesis, applying

$C^{11}O_2$, were also carried out by Frenkel (1941). *Methanobacterium omelianski* was found by Barker and co-workers (1940) to be capable of oxidizing methanol or ethanol to their corresponding fatty acids with the simultaneous reduction of CO_2 to methane. *Propionibacterium pentosaceum* ferments glycerol to propionic acid and water. In the presence of CO_2, succinic acid is also formed (Carson and Ruben, 1940), a result previously obtained by Wood and Werkman (1936). The application of labeled carbon much facilitated the studies of the Wood-Werkman reaction (carbon to carbon addition by C_3-C_1 union). Much of the earlier work of Werkman and Wood has been confirmed by the work of Carson and Ruben (1940), Wood *et al.* (1940, 1941a) and others.

Isotopic carbon was found fixed in the carboxyl group of succinic acid during its formation by the propionic acid bacteria (Wood *et al.*, 1941b). When *Tetrahymena gelii* is cultured in the presence of CO_2, 30 minutes of fermentation suffice to incorporate up to 50 percent of the labeled carbon in carboxyl groups of succinic acid (Van Niel and coworkers, 1942a). It was also shown that, during glycerol or glucose fermentation, the propionic acid contained carboxyl isotopic carbon (Carson and Ruben, 1940; Carson and associates, 1941; Wood *et al.*, 1941a and c). Nishina and co-workers (1941) isolated crystalline derivatives of malic and fumaric acids from preparations of *B. coli communis,* fermenting glycerol and glucose in the presence of $C^{11}O_2$. The metabolism of CO_2 in microorganisms was studied, among others, by Carson *et al.* (1941) and by Slade and associates (1942). Van Niel and associates (1942b) gave a survey of the metabolic significance of CO_2 in microbiological processes, while a report by Krebs (1943) contains a summary of microorganisms whose metabolism has been studied with the use of isotopic carbon.

The problem of interaction of acetate and acetoacetate with the acids of the tricarboxylic acid cycle has been studied by using C^{13} (Buchanan and coworkers, 1945), that of the conversion of acetic acid into glycogen by Lifson *et al.* (1945a). In some of their investigations, acetic acid of which both carbon atoms were labeled was employed. Rittenberg and Bloch (1944, 1945) used acetic acid containing C^{13} in the carboxyl position and deuterium in the methyl group. They found convincing evidence that acetate is converted into glutamic and aspartic acid in the Krebs cycle. Evidence of importance as to the formation of ketone bodies was brought by Medes *et al.* (1945a, b, and c) and Weinhouse *et al.* (1944, 1945). They studied the formation of acetoacetic acid from carboxyl-labeled acetic acid by a variety of tissues *in vitro*. The very extended application of isotopic tracers in the study of acetic acid formation is described in the reviews of Buchanan and Hastings (1946), of Wood (1946) and of Bloch (1947).

The enzyme systems involved in the Wood and Werkman carboxylation reaction have been studied by Werkman and associates, and by Evans and coworkers. The reversibility of C_3 and C_1 addition by oxalacetate β-carboxylase was shown, using heavy carbon, by Krampitz, Wood and Werkman (1941). An important contribution towards the proof that animal tissue fixes carbon dioxide by oxalacetate β-carboxylase was brought forward by Evans *et alia* (1943) when they obtained a cell free preparation from pigeon liver containing this enzyme. The enzyme was heat-labile and catalyzed the decarboxylation of oxalacetate to pyruvate (Krampitz and Werkman, 1941) just as did the bacterial enzyme, but it was activated only by Mn^{++} in contrast to the last mentioned enzyme which could be activated by Mg^{++} as well.

Utter and co-workers found (1945), using heavy carbon as an indicator, that pyruvic acid may be formed from acetyl phosphate and formic acid, a C_2-C_1 union.

Recently Strecker and associates (1948) demonstrated that acetyl phosphate was not involved as a reacting component in the "phosphoroclastic" equilibriums. They labeled acetyl-phosphate in the carboxyl atom contaning C^{13} and got no label in the pyruvate under conditions where C^{14} labeled formiate gave carboxyl labeled pyruvate. This investigation demonstrates the usefulness of double labelling. By making further use of labeled carbon, considerable evidence, in addition to that previously mentioned, has accumulated which points to the importance of the Wood-Werkman reaction in the metabolism of mammals. Liver slices in the presence of isotopic CO_2 were found to produce urea containing a high concentration of carbon isotope (Evans and Slotin, 1940; Rittenberg and Waelsch, 1940). Evans (1940) found also that minced pigeon liver is capable of synthesizing its own dicarboxylic acids from pyruvate. Evans and Slotin (1941) furthermore concluded from their observations that citric acid cannot be a direct intermediate in the formation of α-keto-glutaric acid from pyruvic acid by liver mince and that a carboxylation reaction does not take place in minced pigeon muscle. These observations were extended by the work of Wood *et alia* (1941).

The fact that C^{11}, which is short-lived, could be applied with so much success in the study of glycogen synthesis is due partly to the rapidity with which glycogen formation takes place in the liver after the administration of glycogenic substances, and also to the fact that the preformed stores of liver glycogen can be reduced to a very low level by fasting, so that the composition of the glycogen isolated after the administration of an isotopic glycogenic substance directly reflects the composition of the compounds from which it originates. According to a statement made by Hastings (Buchanan and Hastings, 1946), it was Vennesland who suggested

the possibility of CO_2 incorporation in glycogen and proposed an experiment with $C^{11}O_2$ and non-radioactive lactate. The experiment led to the result (Solomon *et al.*, 1941) that, when administering labeled bicarbonate to rats, an appreciable quantity of C^{11} was found in the glycogen which was formed in the liver. Incorporation occurs to an appreciable extent only when the animal is actually making and depositing glycogen. Experiments of Wood and associates (1945a), applying C^{13}, proved that the CO_2 carbon occupies position 3 or 4 in the glucose derived from newly formed glycogen.

To what extent administered lactate molecules comprise the glucose units of glycogen was cleared up by administering isotopic lactate with radioactive carboxyl carbon and with radioactive α- or β-carbons (Cramer and Kistiakowsky, 1941; Conant and co-workers, 1941; Vennesland *et al.*, 1942a). The results of further experiments (Vennesland *et al.*, 1942b; Buchanan *et al.*, 1942a and b) suggest that the ingested glucose comes into equilibrium very rapidly with the dicarboxylic acid pool before its conversion to glycogen. Using carboxyl-tagged glycine, Olsen and associates (1943) found only a minute part of the carbon of the newly formed glycogen to originate from the administered glycine carbon. The formation of ketone bodies was studied by using C^{13} by Swenseid *et alia*, 1942, in addition to the work of Weinhouse *et alia* (1944, 1945) and Medes *et alia* (1945). Buchanan *et alia* (1943) studied the mechanism of short chain fatty acids *in vitro* with isotopic carbon.

The application of labeled carbon helped greatly in arriving at a reasonable scheme of the total oxidation of fatty acids and in clearing up the relationship of fat and carbohydrate metabolism. In the studies of glycine metabolism in different species with C^{13}, the large-scale conversion of glycine carboxyl carbon to $C^{13}O_2$ was noted in mice and *Torulopsis utilis*, but not in surviving cat heart (Olsen *et al.*, 1943; Lorber and Olsen, 1946; Ehrensvärd *et al.* 1947). Methionine with the carbon isotope, C^{13}, in the β- and α-positions and with the sulfur isotope, S^{34}, was fed to rats by du Vigneaud and associates (1944). Their results demonstrate the formation of cysteine from methione, the transfer of methionine sulfur to a C_3 chain. It should also be mentioned that the short lifetime of C^{11} did not prevent its application in the investigation of the effect of CO upon respiratory metabolism in humans (Tobias *et al.*, 1945).

Application of C^{14}

The availability of C^{14} became a great event in the history of tracer research, as this isotope greatly facilitates the application of radiocarbon in those investigations in which a small percentage of the labeled carbon administered is to be determined. We can easily measure with a Geiger counter five or even less disintegrations per minute, per milligram of carbon. With a starting activity for approximately two percent C^{14} of about 2×10^8 disintegrations per minute, per milligram, the allowable dilution for this carbon becomes approximately 4×10^7. This figure should be compared with the 500 to 5000-fold dilution possible with the use of C^{13}.

Radioactive measurements are furthermore much easier to carry out than mass-spectrographic determinations. The difficulties due to the absorbability of the β-rays of C^{14} in the sample to be measured are not to be overrated. While any sample obtained, without being combusted to CO_2, can be tested by making use of the counter or a similar device, mass-spectrographic determination requires the carbon to be first converted into a form suitable for the measurement. Discovered by Ruben and Kamen (1940a), C^{14} became available at a later stage when its production by the uranium pile was initiated. Barker and Kamen (1945) and Barker *et alia* (1945, 1946) were the first to use C^{14} labeled carbon in the studies of fatty acid metabolism in Clostridium and Butyribacterium. The synthesis *in toto* of acetic acid from C_1 fragments in equilibrium with CO_2 was demonstrated as occurring during glucose fermentation by *Cl. thermoacetium*. Barker and co-workers (1947) determined later the fixation of $C^{14}O_2$ into glycine and acetic acid during the fermentation of uric acid by *Clostridium cylindrosporum*.

Tyrosine labeled with C^{14} in the β-position was injected into rats by Greenberg and associates (1947) and into mice by Reid (1947), and the distribution of C^{14} in various organs was followed. 9, 10-labeled dibenzanthracene has been synthesized and its fate, following administration to mice, has been examined (Heidelberger and Jones, 1947). At the time being, numerous investigations of this type are being carried out by a great number of workers.

To mention further applications of C^{14}, Vennesland *et alia* (1947), applying C^{14}, found the exchange reaction between CO_2 and β-carboxyl carbon atoms of oxalacetic acid to be stimulated by adenosine-triphosphate, but not however by triphosphopyridine nucleotide. The biological precursors of uric acid carbon (Buchanan and Sonne, 1946; Sonne *et al.*, 1946; Shemin and Rittenberg, 1947) were determined by administering labeled compounds to pigeons. Gurin and Delluva (1947) used in their studies on adrenaline precursor, DL-phenylalanine labeled with C^{14} in the carboxyl or α-carbons.

The metabolic lability of the methyl group in methionine has recently been demonstrated by Melville and associates (1947). Winnick *et alia* (1948) brought further evidence that the peptides are important intermediates in protein synthesis. C^{14} was used to show that carbonate, when incorporated into liver slice protein, under certain conditions resides almost exclusively in the carboxyl groups of dicarboxylic acids (Anfinsen and associates, 1947). The very appreciable number of organic com-

pounds containing C^{14} are reviewed in a recent report by Miller and Price (1947).

C^{14} was applied in numerous studies aiming at the elucidation of the process of photosynthesis. Kamen and co-workers (1947) studied the uptake of CO_2 in the dark under the influence of cyanide. In the studies of the assimilation of CO_2 by the plant, C^{14} found an extended field of application. Five-minute dark uptake of $C^{14}O_2$ by Chlorella already equilibrated with CO_2 was found in the dark to form succinic acid containing 70 percent of the added C^{14} (Benson and Calvin, 1947; Calvin, 1948). The utilization of acetate by tobacco leaves, using C^{14} labeled acetate, was studied as well (Barker, 1948). The distribution of C^{14} within the glucose secured from barley seedlings exposed to $C^{14}O_2$ was determined by Utter and Wood, 1946, while Aranoff *et al.* (1947) studied the distribution of C^{14} in photosynthesizing barley seedlings.

In trying to sketch the main lines of development of the application of isotopic tracers in biochemistry, I have had to restrict myself to the discussion of a fraction only of all the impressive work carried with isotopic indicators.

In the course of the 25 years which have elapsed since the first application of isotopic indicators in biochemistry, tracers have been ever increasingly used. The results obtained corroborated in many cases the earlier findings of biochemistry, demonstrating, for example, as clearly and as convincingly the difference between the behavior of intra- and extracellular elements discovered in classical physiological studies. In many other cases, hitherto unknown features of biochemical and physiological processes were brought out, as for example the existence of a new type of permeability which could be denoted as "interchange" permeability in contrast to the "accumulation" permeability which can be measured by classical methods. The problem of the precursor of biochemical products has been tackled in many cases, most remarkable results being obtained, as in the case of creatine formation which was elucidated in all its details. Isotopic tracers on an ever increasing scale find application in every branch of biological sciences.

Possibly the most important result obtained has been the discovery of the dynamic nature of the body constituents. Both the application of stable and of radioactive isotopes has brought out the remarkable rate at which most types of molecules building up the organism are degraded and rebuilt. The determination of the rate of renewal of the body constituents has been the subject of numerous investigations. While many important results have been obtained, we are ignorant about the deeper significance of these incessant renewal processes. One may argue that each molecular renewal is an indispensable step in one or more metabolic processes going on in the organism. Each lecithin molecule formed during such a renewal process in the liver, for example, could be an indispensable step in the oxidation or transport of a fat molecule. Available evidence is in disfavor of this explanation of the lecithin turnover, though it is at present not possible to arrive at an unambiguous result, the precursors of lecithin formation being unknown. One may, however, interpret the renewal processes differently by assuming that *each* turnover is not necessarily a step in a specified biochemical process, just as each sodium ion passing the placenta is not necessarily involved in the formation of the nutrition of the fetus. The placenta has to be permeable to sodium, and sodium being in a comparatively large amount present in the indispensable circulating fluid, sodium passes in very much larger amounts in the time unit than the placenta can utilize. Or, to give another example: in view of the role of zinc in insulin formation, the pancreatic cells must be permeable to zinc, but the large percentage of zinc introduced into the circulation, which is found to rush into the pancreatic cells and to move out swiftly soon again after the zinc concentration of the plasma decreased, can hardly have a direct significance for the function of pancreatic cells. According to the last mentioned view, while the organism is built up on a dynamic pattern which may much facilitate its biochemical tasks, *each* turnover of a constituent is not necessarily an indispensable step in one of the biochemical processes going on in the body. Future development can be expected to shed light on this question of great interest.

Though the topics of the addresses given in this symposium cover only a fraction of the fields in which isotopic tracers found application, they include the discussion of a very great number of important problems. We who have the privilege of participating in this meeting feel deeply indebted to the Biological Association of Cold Spring Harbor and to the leader of this conference, Dr. Demerec, for having given us this unique opportunity to enlarge our knowledge in the field of isotopic tracers and to meet highly esteemed colleagues.

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DISCUSSION

RITTENBERG: Modesty on the part of our speaker has prevented him from properly evaluating the importance of his contributions to the discovery and development of the tracer technique. His original experiments on the transport of lead outlined all the principles which are now so widely employed in the study of biological systems. Only the lack of suitable isotopes prevented the immediate application of this technique to many problems. It is clear that Hevesy understood the scope of the technique, for shortly after D_2O became available he returned to the biological field, studying the rate of water excretion in man. Of his more recent contributions nothing need be said, for we are all familiar with them.

Hevesy, to employ terms suitable to a biological science, was not only one of the fathers of the isotope technique but also the attending gynecologist.

STUDIES ON THE PHOSPHATE METABOLISM OF SOME UNICELLULAR ORGANISMS

MARTIN D. KAMEN AND S. SPIEGELMAN

I. INTRODUCTION

It is now widely appreciated that the metabolic significance of phosphate derives from its participation in esterification reactions (phosphorylation) which may be utilized in biochemical processes both for storage of energy and for specific syntheses of cellular components. The formation of phosphate esters is mediated in the living cell by enzymes (phosphoferrases, phosphorylases, phosphatases) which together with a wide variety of phosphate-containing substrates make up a highly organized complex of chemical systems through which a constant flux of metabolites is maintained in the living cell. In resting and mature cells, regulation mechanisms produce a steady state condition in which overall chemical composition is essentially constant. Experimental examination of metabolic reactions in such systems requires use of techniques which serve to distinguish chemical species while no gross changes in chemical composition are occurring, a situation demanding the employment of isotopic tracers. In what follows we will be concerned with a description and evaluation of tracer researches using labeled phosphate to investigate various aspects of phosphate metabolism in relation to synthetic processes in living cells. This necessitates consideration of the limitations basic to tracer methodology and inherent in the analytical procedures employed.

The work reported includes researches on uptake and turnover of phosphate in a heterotrophic organism, yeast (*Saccharomyces cerevisiae*), two autotrophic organisms—the algae, *Chlorella pyrenoidosa* and *Scenedesmus* D_3 (Gaffron)—and a photoorganotrophic representative from the family Athiorhodaceae, *Rhodospirillum rubrum*. The use of this variety of organisms was dictated by a desire to provide data of a comparative biochemical nature on phosphate metabolism. These researches have been made possible by the fruitful collaboration of a number of colleagues. The work on yeast has been performed with Messrs. E. Juni, M. Sussman and Dr. J. M. Reiner under a grant from the American Cancer Society. The researches dealing with the photosynthetic algae and bacteria were done by one of us (M.D.K.) in colloboration with Mr. H. Gest.

II. TREATMENT OF ORGANISMS PRIOR TO CHEMICAL FRACTIONATION

The nutritional aspects of phosphate metabolism have not been systematically investigated. Much scattered information is available incidental to experiments on biochemical transformations in a wide variety of organisms. One may recall, for example, work on the mold, *Aspergillus niger* (Mann, 1944), in which it was found that the type of growth medium used markedly affected characteristics such as the rate of phosphate utilization, the phosphate content, the rapidity and extent of growth, the respiratory quotient, vitamin content, etc. Similarly in the propionic acid bacterial fermentation of glycerol, it was noted that phosphate, among other factors, influenced the rate of formation of succinic acid and CO_2 fixation (Wood and Werkman, 1940). Very little data is available on the modification of intracellular phosphate distribution by procedures employed in freeing organisms of nutrient media prior to chemical fractionation. In this section there will be reported observations on this aspect of phosphate metabolism.

To determine the effects of washing procedures on phosphate composition, the organisms were grown in media containing labeled inorganic orthophosphate. The compositions of the media were fixed for each organism, the phosphate content being varied for different experiments. The culture conditions have been described elsewhere and need not be detailed here (Juni, Kamen, Spiegelman and Reiner, 1948; Gest and Kamen, 1948; Spiegelman and Kamen, 1947). The organisms so obtained were internally labeled and served as convenient test objects for the determination of phosphate interchanges between wash solutions and cells.

Two types of wash solutions were chosen: (1) normal saline, because of its frequent use as noted in the literature, (2) the particular growth medium employed.

A typical experiment is summarized in Table 1. Five day old and four day old cultures of the algae, *Chlorella pyrenoidosa*, were used respectively in experiments A and B. In the experiments (C and D) on Rhodospirillum, the cultures were five days and eight days old, respectively. The cells were harvested by rapid centrifugation at 0° C. In all cases, the saline solution was 0.85 percent in NaCl. The "medium" wash in the Chlorella experiments was a part of the growth medium containing 50 μg. P/ml. unlabeled, and lacking only in the carbonate of the original complete growth medium. For Rhodospirillum, the "medium" wash was the same as the complete growth medium (700-800 μg. P/ml.) except that the phosphate was unlabeled. The washes consisted of 25 ml. portions of ice cold solution, the successive washes being separated from the residual cells by rapid centrifugation at 0° C. Practically no illumination of the organisms occurred and metabo-

lism was at a very low level during washing. The quantities of cells washed did not exceed 0.3 ml. wet volume in any case.

The quantity of P^{32} appearing in successive washes should have dropped sharply in each case by at least a factor of 100. It is seen that this is not true. Phosphate from inside the cells continued to leak out slowly from one wash to the next, the factor of dilution between washes being usually between 2 and 5. The amounts appearing in the wash were much too large to be accounted for by simple desorption. Experiments C and D indicate a marked effect of age in the washing behavior of Rhodospirillum, the older cultures appearing to leak less phosphate relative to total cellular phosphate. The effect of phosphate in the external wash on the internal P leakage appears to be negligible in Chlorella, but rather marked in Rhodospirillum.

It was evident that successive washes gradually removed internal phosphate despite the low metabolic level of the cells during the washing procedure. To further examine the effects of washing on phosphate distribution washed bacteria from experiment C of Table 1 were suspended in 15 ml. of saline and one ml. aliquots extracted with cold trichloracetic acid (final concentration 5 percent at 0°C for one hour). Equal quantities of cells washed with saline or with medium were used (87 mg. dry weight). In Table 2, the results are summarized. From the specific activity of the P in the external medium and the total activity associated with the cells originally (washes plus washed cells) one could estimate accurately the amount of P^{31} initially present in the bacterial suspension as 177 μg./ml.

It was also noted from the specific activity of the non-acid extractable P that no exchange had occurred between this fraction and either the intracellular extractable P or the exogenous wash P. The total P removed from the cells by saline washes appeared to come from the acid extractable fraction. One ml. of such a bacterial suspension contained only 75.7 μg. P out of an original 177, so that ~100 μg. P had been leached out of the extractable P by saline washes. This amounted to ~87 percent of the P originally present in this fraction. For the medium washed cells, there was observed a *net increase* of 49 percent in this fraction, on the basis of a similar calculation using the data of Table 2. This increase occurred despite a considerable leakage of internal phosphate as evidenced by the appearance of P^{32} in the wash solutions. The rapid interchange of extracellular and intracellular phosphate established in these washing experiments could be exploited for the differential labeling of organisms so that flow of phosphate between the intracellular extractable and nonextractable phosphate could be studied.

Algae which had been washed free of "excess" phosphate appeared to retain normal photosynthetic and respiratory activity as measured by the usual manometric methods. It was found that this dispensable phosphate could be eliminated by growing algae in a medium containing very little inorganic P, in fact as little as 2 μg./ml. compared to the normal (40-50 μg./ml.). Typical distribution data are shown in Table 3. Most of the "washable" P appeared to be "inorganic" (see section III).

Organisms grown in the low P medium depleted

TABLE 1. LEAKAGE OF P^{32} FROM INTERNALLY-LABELED CELLS OF *Chlorella pyrenoidosa* AND *Rhodospirillum rubrum* (SI) INTO SUCCESSIVE WASHES OF SALINE AND GROWTH MEDIUM AT 0°C (COUNTS/MINUTE)

	Chlorella pyrenoidosa				*Rhodospirillum rubrum* (SI)		
	Exp. A		Exp. B		Exp. C		Exp. D
	Saline	Medium	Saline	Medium	Saline	Medium	Medium
Wash 1	625,000	326,000	1,040,000	800,000	382,000	117,000	330,000
Wash 2	401,000	179,000	343,000	425,000	117,000	8,350	40,600
Wash 3	162,000	140,000	35,400	77,500	36,000	5,130	17,100
Wash 4	58,500	88,000	21,000	32,000	12,800	5,650	15,000
Wash 5	22,800	80,500	16,100	16,000	1,800	5,450	10,200
Washed organisms	2,420,000	2,850,000	1,847,500	1,806,000	374,000	683,000	1,125,000
Original supernate c./m./ml.	238,800	238,800	410,000	410,000	232,000	232,000	166,000

TABLE 2. DISTRIBUTION OF P^{31} AND P^{32} IN *Rhodospirillum rubrum* (SI) AFTER WASHING WITH SALINE AND MEDIUM

	Saline-washed		Medium-washed	
	μg.	c./m./μg.	μg.	c./m./μg.
Total P†	75.7	329.0	321.5††	141.7
P in TCA extract	15.3	326.0	254.5	108.5
P in TCA residue	60.4	335.0	51.8	345.0

† Direct determinations—per ml. of suspension.

†† Of this 321.5, 81μg. of specific activity 6.5 were *not* associated with the cells.

Specific activity in original supernate 356 c./m./μg. (651 μg. P^{31}/ml.)

it completely of phosphate. Of course such concentration effects have been known throughout the whole plant kingdom for some time (Krogh, 1946).

It is instructive again to recall the observations on *Aspergillus niger* (Mann, 1944) which parallel those reported for the algae. Thus, molds grown in 0.2 to 0.5 percent K_2HPO_4 media contained 1 to 2 percent dry weight P whereas molds grown in 0.02 percent K_2HPO_4 media had only 0.3 percent dry weight P content. For the latter case, there was again complete depletion of P in the nutrient medium.

TABLE 3. P^{31} DISTRIBUTION IN *Chlorella pyrenoidosa* GROWN IN "LOW" AND "HIGH"-PHOSPHATE MEDIA

Medium	Low P	Low P	High P	High P
Age of Culture	5 day	5 day	5 day	5 day
Exp. No.	1a	1b	2a	2b
Treatment	Not washed	Washed*	Not washed	Washed*
P in TCA ext. (μg.)	41.2	32.0	149.0	90.3
P in TCA res. (μg.)	183.0	191.0	404.0	374.0
P in Wash 1 (μg.)	—	0	—	16.8
P in Wash 2 (μg)	—	—	—	17.2
P in Wash 3 (μg.)	—	—	—	10.7
Total P (μg)	224.2	223.0	553.0	509.0

* The algae were washed with 3–10 ml portions of water at room temperature (approx. 0.2 cc. wet volume of algae in each series).

The mechanism for storage of "inorganic P" has not been elucidated. However it is by no means certain that intracellular inorganic P exists except as an artifact produced by chemical procedures employed to extract cellular constituents. This point will be discussed in more detail in section III.

Numerous examples of P-leakage from cells to medium can be found in data reported for various types of bacteria. In *S. aureus* and *Strep. hemolyticus* both N and P are released into the supernatant liquid in appreciable quantity compared with that extracted by trichloracetic acid (TCA) (Hotchkiss, 1944). Relatively large amounts of both inorganic and ester phosphate are soon found in supernates when *Strep. faecalis* is suspended in saline in the absence of glucose (O'Kane and Umbreit, 1942). During endogenous respiration resting cells of *Thiobaccilus Thiooxidans* release inorganic P into the medium (Vogler and Umbreit, 1942; LePage, 1942). Leakage of P explains the failure of washed *B. coli* to ferment glucose, because addition of inorganic P restores activity (Gale, 1947). Leakage of P from *B. coli* is found to occur in young cultures after two washings with saline in the absence of glucose (Macfarlane, 1939). Most recently, *Trypanosoma equiperdum* has been found to lose appreciable amounts of P when washed with saline (Moraczewski and Kelsey, 1948).

The leakage phenomenon, however, is not universal. Yeast appears to release negligible quantities of phosphate. When grown in P^{32} media only 1 to 2 percent of the total P^{32} content was lost into unlabeled nutrient solution in 24 hours at 20° C (Hevesy and Zerahn, 1946). However these yeast cells were washed thoroughly with nutrient media containing phosphate to remove occluded P^{32} before being suspended in the unlabeled medium so that it is possible some leakage during the washing procedure escaped observation. Yeast washed with phthallate buffer (pH 4.5) does not lose appreciable phosphate (Juni, Kamen and Spiegelman, 1948). If yeast is grown in a labeled P medium, then resuspended in phthallate wash medium, the P^{32} appearing in the wash is precisely what would be expected from serial dilution of adherent labeled nutrient medium, in contrast to the slow leakage reported above for the algae and bacteria. In distilled water, yeast appears to release only about 10 percent of its total P content over a period of several hours at room temperature (Reiner, 1948).

The factors which govern release of P from cells are as little understood as those which dictate the storage of excess phosphate during growth in media containing P in concentrations greater than the minimal requirement. The data presented in this section indicate that, aside from whatever insight can be obtained into cellular metabolism by investigation of washing procedures, it cannot be assumed that cells are inert to such procedures without possible vitiation of interpretations based on data obtained from subsequent manipulations.

III. CELLULAR FRACTIONATION WITH PARTICULAR REFERENCE TO ORIGIN OF INTRACELLULAR "ORTHOPHOSPHATE"

A typical metabolic experiment involves growth of the organisms in a nutrient medium, segregation and resuspension in an experimental medium and finally chemical fractionation of the organisms. Uncertainty in interpretation of chemical data may arise at any point in this chain of operations. We have discussed some of the uncertainties inherent in culturing and segregating organisms. We may now turn to what is perhaps the most important aspect of metabolic investigation, the chemical procedures employed in obtaining cell fractions. It is evident that a major uncertainty involves the extent to which any chemical procedure reproduces the actual chemical content of the cells. The availability of tracer techniques with their potentially enormous sensitivity emphasizes the difficulties inherent in obtaining significant chemical fractions.

The role of chemical fractionation is best discussed in connection with a specific problem—the mechanism whereby externally placed inorganic phosphate enters into the intracellular phosphate cycle. In general two types of mechanism have been proposed. One mechanism is based on the notion that

exogenous phosphate enters as inorganic orthophosphate by diffusion and mixes with intracellular orthophosphate. This phosphate is assumed to be the source from which various organic phosphates in the cell derive phosphate. Another mechanism supposes that entry of phosphate involves esterification at the cellular interface. Intracellular inorganic orthophosphate would then arise primarily from the breakdown of organic phosphate.

It would appear that in principle a choice between these two proposals could be made by use of labeling techniques in which the specific isotopic

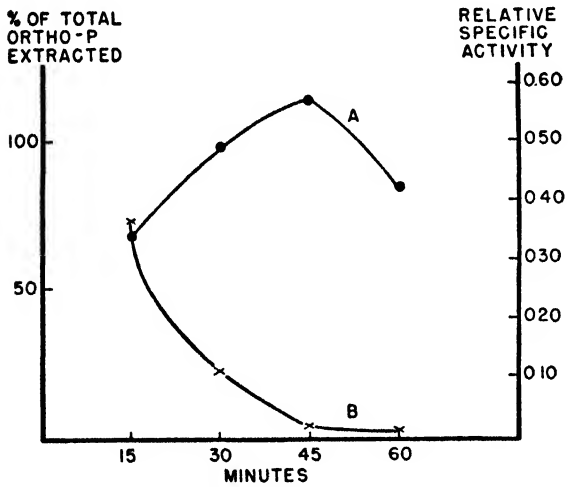


FIG. 1. Heterogeneity of intracellular orthophosphate extracted with cold TCA, as evidenced by variation in specific activity during serial extractions.

Curve A. Specific activity relative to exogenous labeled phosphate as function of serial extraction.

Curve B. Percentage of total intracellular orthophosphate extracted corresponding to each point of Curve A.

content of "ortho" and "organic" phosphate fractions were compared following suspension of cells in a medium containing labeled inorganic orthophosphate in which metabolism was permitted to occur under a variety of experimental conditions. There arises, however, the question of what to regard as cellular "ortho-P." At present the most popular extraction procedure involves the use of cold trichloracetic acid (TCA) with subsequent precipitation of the resulting mixture with magnesia in alkaline pH, this precipitate being considered to represent cellular "ortho-P." The extent to which this is true has still to be assessed.

Another uncertainty is involved in the identity of the organic cellular phosphate with which the labeled content of such "ortho-P" is to be compared. Such comparison requires isolation of the organic P in a reasonably pure state. One is never certain that there does not exist an organic P fraction as yet undetected either because of lability or low concentration which has a higher isotopic labeled content (specific activity) than any of the other cellular fractions, including the orthophosphate.

Some investigations (Juni, Kamen and Spiegelman, 1948) were made on the significance of the ortho-P fraction prepared by the usual TCA extraction and magnesia precipitation method. It had been noted (Spiegelman and Kamen, 1947) from the variation with time of the relative specific activity of all cellular fractions referred to exogenous phosphate that no cellular phosphate, including ortho-P, ever fully equilibrated isotopically with exogenous phosphate. Thus, in yeast cells fermenting glucose in the presence of labeled phosphate, a rapid rise in the relative specific activity of cellular ortho-P occurred in the first 30 minutes, the value reached representing 20 percent of the equilibrium value, after which no further significant increase in specific activity could be noted. One explanation for this lack of equilibration could be that the method of cold TCA extraction used for preparing the orthophosphate fraction actually created a portion of it by hydrolysis of labile organic phosphate esters. From this point of view, the 20 percent which did equilibrate rapidly could either be the original orthophosphate or that which formed by hydrolysis. In any event, this phenomenon indicated that the phosphate fraction, prepared in this manner, was not derived from a homogenous cell fraction. One might also suppose that the ortho-P was made up of inorganic phosphate originating from different sources, analogous to what was found with metaphosphate (Juni, Kamen, Spiegelman and Wiame, 1947). Some of these might be actively involved in the phosphate cycle, whereas others, because of intracellular steric effects, could not enter appreciably in the phosphate metabolism.

Whatever the explanation, it is clear that the assumption of the homogeneity of the ortho fraction prepared by TCA extraction is questionable. The validity of this assumption is made even more doubtful by further experiments which examined the homogeneity of the fraction in another way (Juni, Kamen and Spiegelman, 1948). The usual procedure in preparing the ortho fraction involves a cold TCA extraction for about one hour. An examination was made of the specific activity of the ortho fraction removed in different intervals during an hour's extraction. These experiments were performed with yeast suspensions which were allowed to ferment glucose in the presence of tracer phosphate for one hour. At the end of this time they were put into contact with 5 percent TCA at 5° C. At the end of 15 minutes the yeast was separated by centrifugation and a new addition of cold 5 percent TCA made. This type of serial extraction was continued for 60 minutes. The orthophosphate in the four TCA extracts thus obtained was precipitated and the specific activity determined. The results are given in Figure 1. The lower curve presents the percent of the total orthophosphate extracted in each 15 minute

interval. The upper curve gives the specific activity (relative to exogenous phosphate) corresponding to each fraction. If the orthophosphate of a TCA extract were derived from a homogenous mixture, it would be expected that the specific activities of the different aliquots obtained in each time interval would be identical. Actually the specific activity increased for the three 15-minute intervals and then fell sharply. This type of behavior would be expected if a portion of the orthophosphate obtained in this manner actually originated from the hydrolysis of organic phosphates of different specific activities with varying hydrolysis rates.

One further experiment along these lines may be cited in which the comparison was made of the amounts and specific activities of orthophosphate prepared by different extraction procedures. Cells were treated in the same way as in the previous experiment in order to label the orthophosphate fraction. The extractions were performed on aliquots of the suspension in the cold for one hour, using TCA, tyrocidin,* "fixanol" (technical hexadecyl pyridinium) and a procedure in which the cells were frozen with liquid air in phthalate buffer, thawed, and then incubated for one hour at 5° C. The results obtained in these experiments are summarized in Table 4. In each case 1.2 grams of yeast were employed.

TABLE 4. AMOUNTS AND SPECIFIC ACTIVITIES OF INTRACELLULAR ORTHO-PHOSPHATE PREPARED WITH DIFFERENT EXTRACTANTS

In all cases 1.2 gms. (wet weight) of yeast were extracted. 48 hour cultures were washed and suspended in cold phthalate buffer at pH 4.5. An anaerobic fermentation in the presence of labeled phosphate for 1 hour preceded the extractions. All extractions were carried out at 5°C. for 1 hour.

Extractant	Ortho-P in μgm.	Rel. Sp. Act.
TCA	480	30 1
Tyrocidin	350	18.1
"Fixanol"	906	19.2
Liquid Air	850	19.3

It is clear that the amount of orthophosphate obtained by these procedures is markedly different, fixanol and liquid air yielding almost twice that obtained with either TCA or tyrocidin. Of greater interest, however, is the much higher specific activity of the orthophosphate obtained with the TCA extraction procedure. It seems most reasonable that the so-called "ortho-P" fraction is derived from various cellular components in a manner as yet undefined chemically, so that interpretation of turnover data on the basis of cellular "ortho-P" obtained in the usual manner can be accepted only with many reservations, at least as far as yeast is concerned. To date there exists no valid reason for believing that the ortho-P isolated by any procedure of extraction represents an adequate sample of preexisting intracellular inorganic phosphate.

Despite the questionable validity of data on the specific activity of the "ortho-P" fraction isolated from a TCA extract, it is nevertheless desirable to examine the present evidence relating to the problem of phosphate entry.

In order to avoid confusion with the literature on the subject the inorganic phosphate of a cold TCA extract will be referred to as the intracellular ortho-P. Much work on this problem has been done using muscle tissue, a material which unfortunately is disadvantageous in many ways for investigations of phosphate uptake. The existence of a large amount of tissue extracellular phosphate which cannot be easily removed complicates considerably the interpretation of the data obtained. In experiments which examined the distribution of radioactive phosphorus in resting and contracting muscles it was noted (Sacks and Altschuler, 1942) that the specific activities of adenosine triphosphate (ATP), creatine-phosphate (CP) and hexosemonophosphate were in quite a few instances considerably higher than that of the intracellular orthophosphate, so that it was concluded that phosphate entered the cell via organic esterification. However the specific activity of the intracellular ortho-fraction was not obtained directly but by means of a calculation based on assumptions about average phosphate distributions in the tissue which require separate justification. An examination of the protocols in this work appears to indicate that in many instances agreement in duplicate samples was not adequate to establish the conclusions reached.

In other researches (Kalckar, Dehlinger and Mehler, 1944) a direct estimation of the specific activity of the intracellular ortho-P was accomplished using perfusion to get rid of the extracellular tissue phosphate before making a cold TCA extraction from which the orthophosphate and several of the organic phosphate compounds were isolated. In these researches it was found that in general the phosphorus of ATP and CP were lower in specific activity than that of the internal orthophosphate. There is therefore disagreement with the previous work cited, the conclusion being reached that "phosphate enters the muscle primarily by a physical exchange." It is of interest to note however that in this latter work in certain instances the specific activity of the hexose monophosphate was considerably higher than that of the intracellular orthophosphate. In fact it is remarked "it is not excluded that the uptake of phosphate in the polysaccharide molecule is a process of significance in the passage of phosphate from the outside into the muscle cell."

In an investigation of the same problem (Furchgott and Shorr, 1943) using thin mammalian muscle

*The tyrocidin and fixanol were obtained through the kindness of Dr. R. D. Hotchkiss.

slices, in which the extracellular orthophosphate could be washed out and in which fractions were isolated by TCA extraction, the results obtained were in agreement with those interpreted to indicate that phosphate enters the cell primarily by diffusion.

Despite the validity of the objections raised against the experiments cited in support of the esterification mechanism it cannot be said that the general esterification hypothesis has been disproven. Nor can it be said that the proponents of the diffusion mechanism have offered conclusive evidence for their case. Possibly one of the most convincing arguments for diffusion is the demonstration (Furchgott and Shorr, 1943) that at 2° C, where metabolism was suppressed almost to the vanishing point, the intracellular ortho-P fraction had a higher specific activity than any organic phosphate fraction examined. From this it may certainly be concluded that diffusion of inorganic phosphate as such can occur. This experiment does not however indicate how important quantitatively such a diffusion process is in transferring phosphate into the cell. The coexistence of an esterifying mechanism which might be dominant during normal cellular metabolism has not been ruled out and in point of fact is actually supported by other evidence presented by the same authors. They point out that entry of phosphate at 37° C is five to ten times more rapid than at 2° C, an increase in rate hardly to be expected if a simple diffusion mechanism only were operating.

In general, experiments with cell suspensions has served to further support the concept that entrance of phosphate into cells involves mechanisms other than simple diffusion. In the erythrocyte (Eisenman, Ott, Smith and Winkler, 1940), it was found that one could not account for the amount of phosphate transferred across the cell membrane on the basis of simple diffusion and, furthermore, that the temperature coefficient of the process was one characteristic of a chemical reaction rather than a physical exchange. A similarly high temperature coefficient was found by others (Hevesy, Linderstrom-Lang and Nielsen, 1937; Mullins, 1942) for P entrance into yeast cells. Furthermore, there is general agreement that phosphorus is incorporated only during active metabolism.

However in these researches determinations on total cellular phosphorus were made without further fractionation. It might therefore be argued that in non-metabolizing cells, only the orthophosphate fraction could be involved and hence that exchange by diffusion might have been overlooked. This criticism would not however be valid since the internal orthophosphate of the yeast cell as determined by assay of a cold TCA extract is 10 percent of the total phosphate of the cell. If this orthophosphate had exchanged with the exogenous labeled P it could easily have been detected and measured. One must therefore conclude that physical exchange between inside and outside inorganic phosphate does not readily occur in non-metabolizing suspensions of yeast cells.

Another method of examining this problem is to study the effect of various metabolic poisons on the exchange between "inside" and "outside" orthophosphate. If the mechanism is primarily a physical one, it would presumably not be affected by agents which interfere with the various phosphorylation steps of the glycolytic cycle. If, on the other hand, esterification of the exogenous phosphate is a principal part of the phosphate transfer mechanisms, an inhibitory effect on the exchange between exogenous and endogenous orthophosphate fractions should be observed.

TABLE 5. THE EFFECT OF AZIDE ON TURNOVER OF THE ORTHOPHOSPHATE FRACTION ISOLATED FROM A TCA EXTRACT

The values were attained after 1 hour of anerobic incubation with glucose

	Relative specific activity	% of control
Control	20	100
Azide (2.5×10^{-3} M)	1.2	6

Investigations based on this approach were instituted. One of the agents used was sodium azide (NaN_3). This compound can inhibit cellular synthesis of various kinds without interfering with the anaerobic glycolysis of carbohydrate. An analysis (Spiegelman, Kamen and Sussman, 1948) of the effect of this agent on phosphate metabolism revealed that NaN_3 prevents the transfer of inorganic phosphate to the organic fraction which normally accompanies the coupled oxidation of glyceraldehyde phosphate. An examination (Spiegelman and Kamen, 1947) was made of the exchange between exogenous and endogenous orthophosphate during anaerobic fermentation of glucose by yeast cells in the presence of 2.5×10^{-3} M azide. Labeled exogenous phosphate was used and the usual cold TCA extraction and subsequent precipitation with magnesium-ammonium mixture at alkaline pH was employed for examining the specific activity of the internal orthophosphate. A typical result is reproduced in Table 5.

It is seen that after 60 minutes fermentation the inorganic fraction of the control suspension attained a specific activity corresponding to 20 percent of that of the exogenous phosphate. The inorganic fraction of the azide-treated suspension exchanged only to the extent of 1.2 percent, representing an inhibition of 94 percent. Thus, the presence of an agent which is known to interfere with the enzymatic reactions leading to phosphate esterification also interferes with the exchange between internal and external orthophosphate. This observation is most consistent with the supposition that the mechanism of entry of extracellular phosphate involves the

process whereby orthophosphate is incorporated into the organic fraction.

Arsenate is another agent known (Warburg and Christian, 1939) to interfere with the coupled oxidation which leads to the formation of organic phosphates. In many ways the effects of arsenate and azide are quite similar, and a comparison (Spiegelman, Kamen and Sussman, 1948) of azide- and arsenate-poisoned fermentation revealed many properties in common. It was of interest to examine the effect of arsenate on the exchange between the external and internal orthophosphates during fermentation. Since the mechanism of arsenate interference involves competitive interaction with phosphate for substrate, it was expected that the extent of its effect would be influenced by the level of orthophosphate present at the time of its action. Hence, in these studies a comparison was made at different molarities of external phosphate. The results of these experiments are summarized in Table 6.

At M/60 phosphate and above, no inhibitory effects on the exchange were observed. Actually, as may be seen, the presence of 0.02 M arsenate significantly stimulated the exchange at these phosphate levels. However, at M/90 phosphate, the presence of 0.02 M arsenate depressed the exchange between internal and external orthophosphate by 52 percent. Here again, there is evidence to support the notion that phosphate esterification is an important factor in transferring exogenous phosphate to the internal ortho fraction.

Other metabolic inhibitors were also examined for similar effects. Of these, iodoacetic acid (IAA) and sodium fluoride (NaF) are of primary interest. IAA is presumed to act primarily on the triose phosphate oxidase, which controls the principal reaction leading to the formation of organic phosphate bonds. NaF,

TABLE 6. EFFECT OF ARSENATE ON THE TURNOVER OF THE ORTHO-PHOSPHATE FRACTION ISOLATED FROM A TCA EXTRACT

The values were attained after 1 hour of anaerobic fermentation

Molarity of P	Molarity of arsenate	Rel. Sp. activity	% of control
M/60	Control	17.0	—
M/60	0.02	22.4	131
M/60	0.05	18.3	107
M/15	Control	37.2	—
M/15	0.02	47.5	127
M/90	Control	18.5	—
M/90	0.02	8.9	48

on the other hand, acts at a later stage in the cycle of phosphate metabolism. Presumably, therefore, some phosphate esterifications could occur in the presence of NaF, the primary limitation being the amount of reduced coenzyme available. In view of the different actions of the two agents, it was of interest to compare their effect on inorganic phosphate exchange. Concentrations of the agents were used which resulted in complete inhibition of fermentation. The experiments were carried out under anaerobic conditions and the incubation period was one hour. Results obtained are summarized in Table 7 in which the percent exchange of the ortho fraction and of the organic and residue fractions are

TABLE 7. THE EFFECTS OF IODOACETIC ACID AND SODIUM FLUORIDE ON THE TURNOVER OF THE ORTHO-PHOSPHATE ISOLATED FROM A TCA EXTRACT

The values were attained after 1 hour anaerobic incubation with glucose. The concentrations of the agents employed resulted in complete inhibition of fermentation. A control suspension was run in the absence of inhibitor under identical conditions

Agent	Molarity	Percent of control relative specific activities		
		Acid soluble		
		Inorganic	Organic	Residue
IAA	2.5×10^{-4}	11	10	10
NaF	2×10^{-2}	50	10	12

included. The organic fraction refers to the acid soluble phosphate which remains after removal of the orthophosphate. The residue phosphate is that fraction of the acid insoluble portion which remains after extraction of phospholipid with hot alcohol-ether mixtures.

There is little doubt that these agents interfere with the exchange between the internal and external inorganic phosphates. Of the two inhibitors it is clear that IAA is the more effective in preventing the interchange. One cannot, however, be certain that the relatively poor inhibitory capacity of NaF is primarily a consequence of its interference with the fermentation cycle at a stage following the first oxidative step. The effects on the organic fractions are, however, essentially equivalent. A 90 percent inhibition of the exchange in both the organic and residue phosphate fractions is attained with both agents.

These results are again consistent with the supposition that the internal orthophosphate is derived not directly from the exogenous phosphate but from some phosphorylated intermediate, the formation of which is interfered with by IAA and to a lesser extent by NaF.

An experimentally testable prediction may be made from the assumption that the primary mechanism of phosphate transfer into the cell involves an esterification. It is to be expected that the acceptor of this phosphate would be generated either directly or indirectly by the metabolism of carbohydrate. It is not unlikely that an accumulation of this acceptor would occur if cells were allowed to ferment carbo-

hydrate in a phosphate free medium since under such conditions it would not be used up. If this did happen there should result a noticeable stimulation in the rate of phosphate exchange. Experiments were therefore performed which examined the rate of exchange between internal and external ortho-P in yeast cells following short periods (from 3-5 minutes) of fermentation in a phosphate free medium. Again, all such experiments were carried out anaerobically. Control experiments were performed in which the phosphate and carbohydrate were added simultaneously. Samples were removed five minutes subsequent to the addition of the tracer phosphate and extracted in the usual manner. Results obtained in experiments of this kind are summarized in Table 8. It is clear that in all cases a two to three fold increase in rate of exchange of orthophosphate is attained by a previous fermentation in a phosphate free medium. We thus have another bit of independent evidence for the intervention of a phosphate esterifying step between the internal and external orthophosphate.

TABLE 8. EFFECT OF FERMENTATION IN A PHOSPHATE-FREE MEDIUM ON SUBSEQUENT EXCHANGE IN THE ORTHO-FRACTION ISOLATED FROM A TCA EXTRACT

The values given are those attained 5 minutes subsequent to the addition of phosphate. Letters refer to different culture media employed in growing the yeast for the experiment. All those with the same letters were grown in the same medium

Exp. No.	Relative specific activity		
	Control	Exp.	Exp./control
1a	2.4	5.4	2.2
2a	2.5	5.5	2.2
3b	1.6	4.7	2.9
4c	1.7	3.6	2.1
5b*	2.0	6.4	3.2
6d	0.6	1.6	2.7
7d	0.6	1.6	2.7

* The strain used in Expt. 5b was not the same as in Expt. 3b.

We may summarize the information available on the relations between external and internal orthophosphate. The exchange between these two fractions has been found to have the following properties:

1) It has a temperature coefficient too large to be explained on the basis of simple diffusion.

2) It is severely inhibited by agents such as azide, arsenate, and IAA, which are known to interfere with phosphate esterification.

3) It is less severely inhibited by NaF which presumably interferes with the phosphate cycle in a less direct manner.

4) Exchange is markedly reduced in suspensions of cells metabolizing slowly.

5) Pretreatment with substrate in the absence of phosphate accelerates subsequent exchange with phosphate.

6) High specific activity is attained relative to "organic" P.

All of these facts are consistent on the assumption that the primary mechanism of the entrance of phosphate is via an esterifying mechanism and that consequently, the "internal ortho-P" as isolated from a cold TCA extract of yeast is derived principally from the breakdown of organic phosphate compounds.

IV. Uptake and Turnover of Phosphate in Relation to Cellular Syntheses

Results of tracer investigations on the uptake and turnover of phosphate into various cellular fractions of yeast as obtained both in our laboratories and elsewhere have been discussed at some length in previous Symposia (Spiegelman, 1946; Spiegelman and Kamen, 1947). These data will therefore be accorded only brief treatment in this section.

Some aspects of phosphate uptake and turnover with special relevance to the mechanism of phosphate entry have already been mentioned (Section III). In general it has been observed that maintenance of anaerobic carbohydrate metabolism uncoupled from assimilation involves a very small fraction of the intracellular phosphate. Even under conditions in which active assimilation is initiated a relatively major fraction of the intracellular phosphate is inert. As will be seen shortly this statement holds equally well for aerobic photosyntheses in algae and anaerobic photosyntheses in purple bacteria. The flow of phosphate from the acid insoluble cellular phosphate is prevented when assimilation and cellular syntheses in general are inhibited by agents such as azide and dinitrophenol (Spiegelman and Kamen, 1946). The interpretation of these data remains in doubt because of the finding that a major portion of such acid insoluble phosphate in yeast appears to be not entirely nucleoprotein but also protein bound metaphosphate as well as other phosphate as yet unidentified. At present the assumption that nucleoprotein metabolism mediates protein synthesis appears to rest on no better grounds than that it is not in disagreement with much evidence of an indirect nature (Spiegelman and Kamen, 1947).

The mechanism for the peculiar effect of azide in differentially inhibiting both phosphate uptake and turnover as well as assimilation during anaerobic fermentation of glucose by yeast has been investigated (Spiegelman, Kamen, and Sussman 1948) and the following pertinent findings obtained:

1. Esterification of intracellular inorganic phosphate is suppressed at azide concentrations which do not affect the rate of glucose fermentation. Exchange of internal and external ortho-P is also inhibited by the same concentrations of azide.

2. Azide decreases sensitivity of fermentation to

poisons which inhibit the triose phosphate dehydrogenase.

3. Inhibition of fermentation by fluoride is increased in the presence of azide.

4. Arsenate-poisoned cells respond to inhibitors of triose phosphate dehydrogenase and to fluoride in a manner similar to azide treated cells.

The most obvious explanation for these observations is that azide, like arsenate (Warburg and Christian, 1939) replaces phosphate in the coupled oxidation step forming an acyl azide which like the acyl arsenate analogue is highly unstable and spontaneously dissociates thus freeing the system from the phosphorylation requirement of the oxidative enzyme. However, a direct test of this hypothesis, using a reconstructed *in vitro* system of pure enzyme, glyceraldehyde phosphate, and diphosphopyridine nucleotide, has failed to reveal any effect of azide on the rate of reduction of the coenzyme. Nor has any evidence been obtained that a replacement reaction between azide and the acyl phosphate of the diphosphoglycerate occurs. At present it appears that the most likely mechanism involves a splitting of the acyl phosphate when the diphosphoglycerate combines with phosphoferrase. Effectively then azide would act to release the glycolytic cycle from the regulatory restraints imposed by limiting amounts of adenylic acid or adenosine diphosphate acting as phosphate acceptors at the phosphoferrase level. Space limitations prevent a fuller discussion of the azide effect for which the reader is referred to the literature (Spiegelman, Kamen and Sussman, 1948).

When phosphate uptake and turnover are investigated in photosynthetic organisms a set of observations quite similar to those reported for yeast is noted, (Gest and Kamen, 1948) in that induction of cellular synthesis invariably results in increased phosphate esterification. It will be remembered that in yeast inclusion of ammonia as a nitrogen source increases phosphate turnover into all fractions, notably nucleic acid and protein-bound metaphosphate. A similar effect is seen in photosynthetic systems when they are illuminated. Light causes the same effect in algae and purple bacteria that ammonium salts evoke in yeast. However, addition of ammonium salts along with trace elements and growth factors to the experimental medium fails to elicit any marked increase in phosphate uptake or turnover in the algae and bacteria.

Typical results are shown for the organisms Chlorella and Scenedesmus in Figures 2 and 3 where specific activity in the acid insoluble residue is plotted as a function of time in light and dark. (The illumination intensity in these experiments was always near or at saturation). In all the experiments performed no significant differences between light and dark samples with respect to P^{31} content has been detected. It will be noted that the turnover into the insoluble fraction is greater in the light than in the dark. However, the extent of turnover is not directly proportional to the overall metabolic activity. For example, in Figure 3, the rates of O_2 absorption and production observed in control vessels run simultaneously were as follows:

(a) In the absence of KCN, photosynthetic oxygen (evolved) = + 129μl./30 min.; Respiratory oxygen (consumed) = −36.2 μl./30 min.

(b) In the presence of KCN, photosynthetic oxygen = + 37μl./30 min., Respiratory oxygen = −43.5 μl./30 min.

Thus, although the endogenous respiratory rate was slightly greater in the presence of cyanide, the

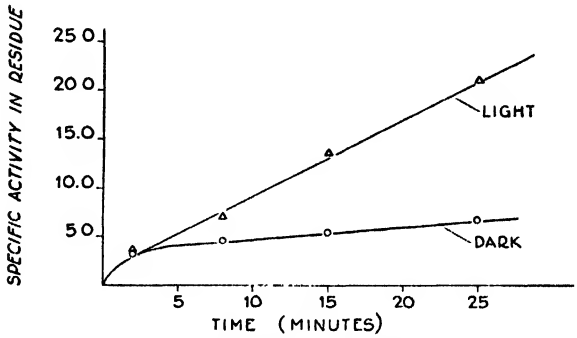
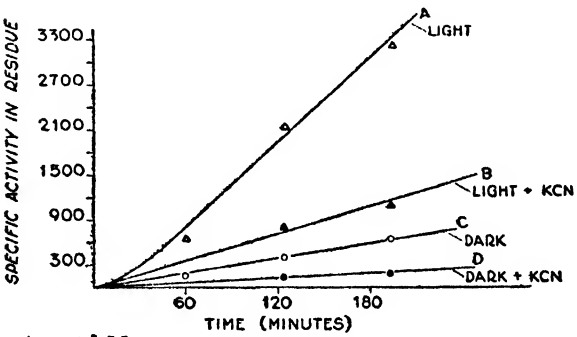


FIG. 2. Effect of illumination on P^{32} uptake into insoluble P fractions by Scenedesmus D_3 algae in 0.1 M $NaHCO_3$ containing 0.16 mg. ortho P/ml. and 0.76 mg. $(NH_4)_2$ SO_4/ml. Temp. = 30°C.



4.5×10^{-3} M.

FIG. 3. Effect of illumination on P^{32} uptake into insoluble P fractions by *Chlorella pyrenoidosa*. Algae in Warburg's No. 9 buffer, temp. = 22°C. Final concentration of KCN =

incorporation of P^{31} into the insoluble phosphate was markedly less than in the absence of cyanide. These results are reminiscent of the results with yeast in the presence of azide which almost eliminates phosphate uptake without changing overall metabolic activity. It is apparent that specific rather than overall metabolism determines the pattern of phosphate uptake.

It should be noted that the stimulation of P uptake in the algae appears to occur in the absence of any nitrogen assimilation in contrast to yeast.

TABLE 9. P^{32} UPTAKE BY *Rhodospirillum rubrum* (SI) IN 0.05% $NaHCO_3$

	Exp. A*					Exp. B†					
	Light		Dark			Light			Dark		
	(a)	(b)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
c./m./μg. P^{31} in residue	159	175	8	8	7	2310	3070	3110	356	299	276
P^{31} in residue (μg.)	154	142	141	148	145	94	91	96	88	90	88

* Exp. A—7 day culture grown in "high" P medium (700–800 μg. P/ml.) with *n*-butanol as H-donor. *N*-butanol was used also as H-donor for the uptake experiment. Gas phase—5% CO_2 in N_2. Temp.—25°. Duration of experiment—230 minutes. Total P^{31} = 297 μg.

† Exp. B—68 hour culture grown in "low" P medium (~40 μg. P/ml.) with acetate as H-donor. Sodium butyrate was used as H-donor for the experiment. In (a), light and dark, no H-donor was added. Gas phase—5% CO_2 in N_2. Temp.—30°C. Duration of experiment—150 minutes. (Cells "dissimilated" by aeration prior to exp.) Total P = 117 μg.

The effect of illumination on P uptake into the insoluble P of *Rhodospirillum rubrum* is more pronounced than in the green algae. Typical experiments are summarized in Table 9. It is seen that a large differential in specific activity between light and dark occurs, even in the cells furnished no substrate (endogenous controls, set (a) of experiment B). In these experiments the rate of autofermentation (endogenous liberation of CO_2 in the dark) was almost equal to the rate of CO_2 assimilation in the light. Yet much greater ratios of specific activities in light and dark products were obtained than in the case of the algae where light metabolism was four to five times greater than dark metabolism. Thus again there is no simple relation found between extent of P uptake and the overall metabolic level.

The role played by phosphorylation in CO_2 reduction cannot be defined without detailed knowledge of the phosphate compounds actively engaged in the turnover of P between the soluble P and other portions of cell phosphate. A partial fractionation of the cellular phosphate is indicated in the data of Table 10 which shows the results of experiments in which bacteria were exposed for 160 minutes in light and dark to a labeled phosphate medium, centrifuged, washed three times with 15 ml. portions of cold saline, and fractionated as indicated. It appears as in yeast that the most complete equilibration with exogenous P is encountered in the soluble P fraction. The extent of equilibration in the algae has been indicated in Table 9. In the experiments of Table 10, the extent of equilibration cannot be determined because of P exchange between cells and medium during the experimental period. The item of most interest is the high P^{32} content of the KOH extract which represents "total protein-P." In contrast to yeast, the KOH residue contains no detectable P^{31}; the P^{32} observed in this fraction may well be owing to contamination from the KOH extract.

Some experiments have been performed to ascertain whether or not light stimulates a true turnover of phosphate. It will be appreciated that the interpretation of P^{32} uptake is complicated by the use of tracer phosphate with high specific activity because under these conditions large amounts of radioactivity are associated with quantities of P^{31} which cannot be detected chemically. Appearance of P^{32} in the soluble and insoluble fractions without a significant change in P^{31} distribution does not necessarily mean that there has been turnover because

TABLE 10. P^{32} UPTAKE AND DISTRIBUTION IN *Rhodospirillum rubrum* (SI) DURING PHOTOSYNTHESIS AND AUTOFERMENTATION*

	Light			Dark			
	I	II	III	IV	V	VI	III/VI
	c./m.	μg. P^{31}	c./m./μg. P^{31}	c./m.	μg. P^{31}	c./m./μg. P^{31}	
P^{32} supernate	374,000	22.9	16,300	2,120,000	45.8	46,250	
Wash 1	27,200	0	—	86,300	0	—	—
Wash 2	8,090	0	—	8,000	0	—	—
Wash 3	7,500	—	—	5,880	—	—	—
TCA extract	550,500	62.5	8,825	284,000	65.5	4,335	2.04
TCA residue:							
Lipid fraction	24,600	125.4	196	8,900	125.1	71.3	2.85
KOH extract	1,355,000	295.5	4,585	102,025	289.5	353.0	13.00
KOH residue	1,860	0	—	375	0	—	—
Total	2,348,750	506.3	—	2,615,480	525.9	—	—

' Culture and conditions similar to those described for Exp. B, Table IX.

the data may be explained by uptake into one or both fractions of minute amounts of P^{31}.

The flow (turnover) between cellular P fractions has been studied by exposing Chlorella to labeled P for a very short time. In this way cells containing soluble P with a high specific activity and insoluble P with a low specific activity were obtained. The cells were then exposed to light and dark in a solution devoid of exogenous P. Flow between the two cellular fractions could then be followed in a system uncomplicated by exchanges between cells and medium. In Table 11 results of such an experiment are shown (in experiment C, exogenous P was present as indicated in the footnote). In these experiments as well as all that have been reported in this paper all values were averages of duplicate or triplicate determinations.

It will be noted that the specific activity in the acid soluble P is higher than the insoluble P. With shorter exposures in the incubation period the differential between the two fractions is enhanced (experiments B and C). In the light, the specific activity of the soluble P decreases significantly more than in the corresponding dark samples.

Thus there is a light stimulated flow of low specific activity P from the insoluble P into the soluble P. A simultaneous flow in the reverse direction is evidenced by corresponding increases in the specific activity of the insoluble P. In experiment C, the stimulating effect of light is apparent in the increased specific activities of the insoluble P. The soluble P, on the other hand shows no corresponding changes. This comes about because of the much higher P^{32} content of the soluble P at the beginning of the experiment. In this experiment ~100 μgm. of the soluble P came from exogenous unlabeled phosphate so that the initial specific activity of the algal soluble P was perhaps as high as three times the indicated value of 2200. Thus, the flow of a few μg. of soluble P into the insoluble fraction could raise the specific activity of the latter as much as 100 percent while an identical flow of insoluble P into the soluble fraction would change the latter only a few percent.

From these data it is a simple matter to calculate the quantity of phosphate participating in the flow during light and dark periods. In experiment A, the amount moved turns out to be 4.4 μg. in the dark and 7.1 μg. in the light. While this represents a very small fraction of the total cellular P, it is a sizable portion of "unwashable" soluble P. Furthermore it will be noted that while the ratio of the final specific activities in the light and dark insoluble P fraction is only 2565/2240 or 1.14, the actual stimulation in flow giving rise to this ratio is 7.1/4.4 or 1.62. Thus, a very large effect on the turnover results in a rather small change in final specific activities of the fractions examined. The significance of this observation with regard to the possibility of overlooking a light stimulated turnover in the algae when examining merely initial and final specific activities of cellular fractions without regard to dilution by "ballast" phosphate which may be present (as in experiment C) need not be labored.

The low equilibration values noted (Table 9) are in good accord with the small amounts of phosphate found to turn over in the experiments of Table 11. It is obvious that the interpretation of simple uptake experiments is rendered difficult by the fact that these small amounts of cellular phosphate are involved, particularly in view of the large amounts of phosphate moved about by washing procedures which in turn are influenced by culture conditions.

TABLE 11. FLOW OF P BETWEEN SOLUBLE AND INSOLUBLE FRACTIONS IN *Chlorella pyrenoidosa*

	TCA extract		TCA residue	
	c./m./μg. P^{31}	μg. P^{31}	c./m./μg. P^{31}	μg. P^{31}
Experiment A*				
Zero time	9985	19.0	1705	68.1
Dark	7810	17.3	2240	67.5
Light	6890	17.3	2565	60.3
Experiment B†				
Zero time	1970	27.5	113	124.5
Dark	1073	30.4	280	117.0
Light	734	34.3	340	115.3
Experiment C†				
Zero time	2175	131.0	159	84.3
Dark:				
80 min.	2245	128.5	189	84.1
150 min.	2200	132.0	196	91.0
Light:				
80 min.	2290	124.8	237	85.5
150 min.	2055	127.0	297	84.5

* Exp. A: 3 day culture grown in "high" P medium. Exposed to P^{32} in Warburg No. 9 buffer under good illumination for 20 minutes at 21°C. Washed cells resuspended in No. 9 buffer at 23° for 120 minutes.

† Exp. B: 2 day culture grown in "low" P medium. Exposed to P^{32} in No. 9 buffer in light for 1 minute at room temp. Washed cells resuspended in No. 9 buffer at 25° for 197 minutes.

† Exp. C: 4 day culture grown in "high" P medium (containing added Fe and trace elements). Exposed to P^{32} in complete medium in light for 2 minutes at room temp. Washed cells resuspended in complete medium at 20° for times indicated. Approx. 100 μg. of the quantities listed as TCA extractable P in this experiment were present as exogenous phosphate.

We may conclude by noting that the data presented on the stimulation of turnover of phosphate appear to be the only clear cut evidence to date that phosphorylation is mediated by light directly or indirectly in photosynthetic organisms. A discussion of the possible mechanisms involved in the coupling of phosphorylation to CO_2 assimilation would take us far afield and in any case is best de-

ferred until more data on the phosphate composition of algae and bacteria are available.

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Discussion

ROTHSTEIN: Dr. Kamen suggests that the transfer of inorganic phosphate across the cell membrane is not a simple diffusion process, but is closely linked to carbohydrate metabolism. Such a connection could imply the incorporation of inorganic phosphate into an organic compound on the surface of the cell, such as that suggested by Sacks for muscle. It is required for this hypothesis that certain enzymes be located on the surface of the cell which are capable of esterifying phosphate.

In support of this view, we have obtained data which show that the surface of the cell is not simply a passive semi-permeable layer, but that it contains a number of active enzymes, particularly those related to the initial steps in the metabolism of external substrates. A series of phosphatases are located on the surface of the yeast cell that can hydrolyze compounds such as inorganic pyro- and tri-phosphate, ADP, ATP, phenyl phosphate, glycerophosphate, hexosemonophosphates, and hexose diphosphate (Rothstein and Meier). Sucrase is also located on the cell surface of yeast (Rothstein and Meier), as is perhaps lactase (Myrback and Vasseur).

Even more closely related to the problem of phosphate transport are the studies of uranium inhibition of glucose metabolism (Muntz, Singer, and Barron, 1948; Rothstein and Larabee, 1948; and Rothstein, Frenkel, and Larabee, 1948). Uranium acts at the surface of the yeast cell, apparently by complexing with pyrophosphate groups involved in the phosphorylation of glucose. This suggests that the enzymes involved in glucose phosphorylation, plus those involved in recharging the phosphate-donating system (ATP?), are located on the surface of the cell. Possibly the transport of inorganic phosphate into the cell involves some of the same surface enzymes that take part in the metabolism of glucose.

HEVESY: Dr. Kamen gave us an impressive presentation of the difficulties encountered when trying to determine the total uptake of labeled orthophosphate by some unicellular systems. Fortunately, when working with animal tissue, conditions are far from being so unfavorable as in some

of the cases investigated by Dr. Kamen. Furthermore, we are often interested not so much in the total labeled orthophosphate content as in its specific activity. In the latter case, leakage does not matter if only sufficient cellular orthophosphate remains in the tissue to permit such a determination.

A serious disturbance in such measurements is what we may call pollution of genuine cellular orthophosphate by orthophosphate of secondary origin, formed by degradation of organic phosphorus compounds in the course of, or previous to, the extraction process. In experiments of short duration, which are of great interest, some of the organic phosphorus compounds show a much lower specific activity than orthophosphate phosphorus. Clearly a partial decomposition of some of the latter, during which orthophosphate of low specific activity is formed, will lead to a decrease in the specific activity of the cellular orthophosphate phosphorus. This disturbing effect was demonstrated very convincingly by Dr. Kamen in his investigations of the phosphorus turnover in some unicellular systems. As to the behavior of animal tissue, it may be mentioned that, if one half of a rat liver obtained a few hours after administration of P^{32} be extracted with cold trichloroacetic acid at once after removal from the animal, its orthophosphate phosphorus is found to show an appreciably higher specific activity than if the extraction takes place some weeks later, even if the liver was kept in the ice-box during that time. This result can hardly be interpreted other than as a pollution of the genuine orthophosphate phosphorus by less active orthophosphate P of secondary origin.

Simultaneously with the determination of the specific activity of orthophosphate P, we often wish to know the specific activity of other P fractions, for example that of the residual P. To obtain residual P a very thorough extraction with trichloroacetic acid, ether-alcohol and so on is carried out. For this reason it is not advisable to apply the trichloroacetic filtrate obtained in the course of such a purification process in the determination of the specific activity of orthophosphate P. The advisable procedure is to extract rapidly with cold trichloroacetic acid the frozen tissue shortly after its removal and use this fraction in the determination of the specific activity of the orthophosphate P, and to discard the further trichloroacetic acid filtrates obtained in the purification of the residual fraction.

In the determination of the rate of renewal of organic acid-soluble phosphorus molecules we have to know the specific activity of orthophosphate P. Often it is, however, more favorable to replace the determination of the specific activity of orthophosphate P by the measurement of the corresponding value of the pyrophosphate phosphorus ($P_{2,3}$) of ATP, or preferably of the terminal phosphorus of ATP. The labile phosphorus of ATP gets into rapid exchange equilibrium with the cellular orthophosphate P, and its specific activity is, in contrast to that of the latter, not affected by a decomposition of some of the organic phosphorus compounds in the course of the extraction process. Even if a large percentage of creatine phosphoric acid present should split off orthophosphate P, the specific activity of the labile P of ATP will remain unaffected, nor will the presence of a non-negligible amount of extracellular orthophosphate influence the labile ATP phosphorus values.

We compared the specific activities of the orthophosphate P of the blood plasma, of the liver tissue, and of the labile P of ATP in a great number of cases in which P^{32} was administered to the rat two hours previously. The ATP labile phosphorus values were almost identical with the plasma orthophosphate phosphorus values, while the tissue orthophosphate values (due to the great permeability of the liver cells to phosphate, the specific activity of the tissue orthophosphate P is almost identical with that of the cellular orthophosphate P) were found to show fluctuations which are presumably due to the above mentioned pollution effect.

COMPARATIVE METABOLISM OF RADIUM, STRONTIUM, AND CALCIUM

WILLIAM P. NORRIS AND WALTER KISIELESKI[1]

Calcium is one of the most abundant minerals in the vertebrate body and, besides constituting a major portion of the skeletal structure, is known to exert a considerable influence in the normal behavior of certain of the soft tissues. Its relationship to the function of the parathyroid glands, and vitamin D and its role in certain pathological conditions such as arteriosclerosis and neuro-muscular disorders have been recognized and extensively studied.

The comparatively recent advent of isotopic tracers, both stable and radioactive, has been of inestimable value in demonstrating the general dynamic state of metabolic reactions. Up to the present time, however, the difficulties involved in the characterization and production of a radioactive isotope of calcium in quantities suitable for biological experimentation have, unfortunately, precluded a great deal of work with this element. It now appears that the only isotope of calcium suitable for biological tracing is that of mass 45, which has a half life of 180 days and emits a beta particle with a maximum energy of 0.26 Mev. The cyclotron and, more recently the chain-reacting uranium pile, have made limited quantities of Ca^{45}, usually in rather low specific activities, available for biological research during the past eight years.

Studies with radiocalcium and radiostrontium have been done by some investigators under various metabolic conditions to determine the degree of similarity in the behavior of the two. Earlier investigations (Kinney and McCollum, 1923; Klein, Becker and McCollum, 1930; Robison, Law and Rosenheim, 1936; Robison and Rosenheim, 1934; Roche and Mourgue, 1939; Shipley *et al.*, 1922; Sobel, Cohen and Kramer, 1935) have shown conclusively that strontium cannot be used as a complete substitute for calcium. Animals fed large amounts of strontium or maintained on diets in which calcium was largely replaced by strontium were observed to develop abnormalities in bone calcification and proliferation of the dentinoid in teeth. This condition is known as "strontium rickets." The replacement of the one percent $CaCO_3$ in the diet of weanling mice with one percent $SrCO_3$ resulted in a marked diminution in growth, so much so that the weights of the mice never exceeded five grams (Pecher, 1942). The actual chemical toxicity of strontium is rather low. Pecher (1942) gave 2.5 grams of strontium as the lactate to each of two eight-pound rabbits and observed no marked effect on the general condition of the animals.

More recent studies utilizing the radioactive isotopes of calcium and strontium have indicated that strontium, in tracer amounts, can be expected to behave qualitatively in a fashion similar to calcium. Good evidence is available to indicate, however, that even in tracer amounts the quantitative behavior of the two is not identical. Thus, Pecher (1941, 1942), in his experiments with calcium lactate (0.8 mg. Ca) injected intravenously into mice, found 58 percent retained after 24 hours, whereas the retention of strontium lactate (1.6 mg. Sr), similarly administered, was 33 percent at the same time. The oral administration of calcium and strontium lactates to mice resulted in 13 to 30 percent and 6 to 14 percent retention, respectively, in the skeleton after two days. The use of smaller doses of inert strontium gave somewhat higher recoveries ranging from 32 to 62 percent. Greenberg (1945) showed that vitamin D in rachitic rats promotes the absorption from the gut and the deposition in bone of both calcium and strontium. This was evident both from the increased radioactive content of bone and the increased urinary excretion of both isotopes. The percentage uptake of calcium was greater than that of strontium in every case.

It has long been recognized that radium is also inclined to follow the general pattern of calcium metabolism. The considerable interest developed in radium metabolism as a result of a number of cases of radium poisoning in the radium dial painting industry led investigators to make a reasonably extensive study of the problem. In addition to descriptions of the biological damage resulting from the deposition of radium in the body (Castle, Drinker and Drinker, 1925; Gettler and Norris, 1933; Martland, 1929, 1931; Martland, Conlon and Knef, 1925; Martland and Humphries, 1929; Reitter and Martland, 1926), sufficient data are available to demonstrate conclusively that radium, several days after administration, is deposited almost entirely in bone and is generally associated with calcium. Evans and his associates (1944) have shown by radioautographic techniques that radium is strongly concentrated in bone and especially in the region of the epiphyses. Analyses of the soft tissues indicated the presence of very little radium in the soft tissues. Aub and Calhoun (unpub.) have shown further, from studies with radium and madder root, that these materials are originally deposited in bone trabeculae from which site they are gradually dis-

[1] The authors are indebted to Mrs. Blanche Lawrence and Miss Lois Woodruff for technical assistance in performing this work.

seminated throughout the bone. Aub and co-workers (1938) have reported that cases of radium poisoning may be improved by treatment with parathyroid hormone, low calcium diet and mild acidosis produced with ammonium chloride—a treatment calculated to increase calcium and radium excretion and to produce some redistribution of radium. Norris, Neal, Jacobson, and Brues (unpub.) found that treatment with parathyroid hormone together with a low calcium diet of a middle-aged patient (Billings Hospital), who had contained a small quantity of radium for several years, increased the daily excretion of radium by at least a factor of ten.

Since it is well recognized that the retention of calcium in the animal body is a function of age, two. This ratio decreased rapidly with time and became fairly constant when the time of injection was about 20 days before birth. At this time the specific activity of the young was only seven percent that of the mother.

The data of Erf and Pecher (1940), Pecher and Pecher (1941), and Finkel (1947) indicate that the injection of calcium or strontium into lactating animals results in the appearance of 10 to 20 percent of the administered dose in the milk in two to four days. The radioactive content of the milk was seen to diminish rapidly with time. The work of Finkel (1947) and Anthony *et al.* (in press) showed that the post-partum retention of female mice injected with $Sr^{89,90}$ was almost identical with that of virgin

TABLE 1. RÉSUMÉ OF REPORTED RETENTION VALUES FOR THE ALKALINE EARTHS

Author	Species	Element	Route of adm.	% Retention	% Excretion Urine	Feces
Greenberg (1945)	Rat	Ca	Oral	31.0 (skeleton)	29.4	32.5
	Rat	Sr	Oral	15.0 (skeleton)	32.6	48.8
	Rat	Ca	I.V.	45.0 (skeleton)	25.0	18.0
	Rat	Sr	I.V.	26.0 (skeleton)	48.8	17.3
Pecher (1942)	Mouse	Ca	I.V.	58.0 (skeleton)	14.0	28.4
	Mouse	Sr	I.V.	33.0 (skeleton)		
	Mouse	Ca	Oral	23.0 (skeleton)		
	Mouse	Sr	Oral	11.0 (skeleton)		
	Rat	Sr	I.V.		23.4	19.4
Campbell and Greenberg (1940)	Rat	Ca	Oral	23.4 (9 hrs.)	65.5	11.0
Armstrong (1945)	Rat	Ca	Oral		2.3	10.8
Armstrong and Barnum (1948)	Rat	Ca	Oral	24.7	2.05	64.5
Greenberg *et al.* (1943)	Dog	Sr	I.V.	(9 days)	20.0	22.0
Anthony *et al.* (in press)	Rat	Sr	I.P.	56.0	40.0	
	Rabbit	Sr	I.P.	21.0	66.0	
	Mouse	Sr	I.P.	45.0	54.0	
Evans *et al.* (1944)	Rat	Ra	Oral	2–10	5–10	81–88
Thomas and Bruner (1933)	Rat	Ra	subQ	25.0		
Dominici *et al.* (1913a and b)	Rabbit	Ra	I.V.	30–55		

nutritional state, and general metabolic condition, it is not surprising to find some discrepancies in retention and excretion values reported by various authors. (A résumé of retention values found by various investigators is contained in Table 1.)

Pecher and Pecher (1941) injected pregnant mice with both Ca^{45} and Sr^{89} as the lactates and observed the deposition of both elements in the foetus. No appreciable calcium or strontium was found in the foetus earlier than the third quarter of pregnancy regardless of the time of injection. The percentage recovery in the young was found to decrease with time since injection. Thus young mice delivered from mothers injected nine days earlier contained two to three percent of the injected dose, whereas with an interval of 21 days or more the retention in the young was considerably less than one percent. Finkel (1947) confirmed these results using Sr^{89}. She noted that the specific activity of young mice, born one to four days after injection, exceeded that of the mother at the time of parturition by a factor of more than mice at comparable times. From 75 to 80 percent of the strontium which would normally have been excreted was found in the young mice.

EXPERIMENTAL RESULTS

The results of the experiments which we should like to report at this time were obtained using groups of male Sprague-Dawley rats of comparable age and weight. The animals varied in weight from 200 to 280 grams and averaged about 250 grams. There was considerably less variation in weight in any single experiment. The rats had access to food and water at all times. The diet, purchased from the Ralston Purina Company, was nutritionally complete and contained 1.17 percent calcium and 0.87 percent phosphorus.

In the various experiments the rats were injected intravenously with either Ca^{45}, $Sr^{89,90}$ (about 75 percent Sr^{89} and 25 percent Sr^{90}) in equilibrium with the radioactive Y^{90} daughter of Sr^{90}, or radium. The radioactive materials were administered as the chlo-

rides in isotonic solutions at pH 3 to 4. Each rat received 0.5 ml. The quantities of radioactivity administered to each rat in the various experiments were 40 μc. of Ca^{45} (1.5 mg. Ca^{40}), 115μc. of $Sr^{89,90}$ (carrier free), and 25 μc. of radium, respectively. Experiments with radium and strontium have shown the retention of these two materials to be significantly less in mice than in rats.

Discussion of Results

Both the urinary and fecal excretion of the alkaline earths under consideration proceed very rapidly.

Table 2. Comparative Values of Excretion and Retention of Intravenously Administered Ca^{45}, $Sr^{89,90}$ and Ra in Rats

Interval	Ca	Sr	Ra
Total Retention (% Injected Dose)			
1 day	94	90	80
3 days	91	85	63
10 days	89	68	57
30 days	80	56	55
Skeletal Retention (% Injected Dose)			
1 day	86	81	73
3 days	88.5	81	58
10 days	87	67	55
30 days	79.2	55	53

Total Excretion (% Injected Dose)

	Urine	*Feces*	*Urine*	*Feces*	*Urine*	*Feces*
1 day	0.6	4.3	5.5	4.7	9.0	10.4
3 days	0.74	8.5	7.6	9.8	12.8	21.5
10 days	0.95	10.8	11.3	15.4	13.8	23.2
30 days	—	—	15.3	22.0	14.4	24.0

This is evidenced by the fact that appreciable portions of the injected dose may be found in the intestines, kidney, and bladder as early as one minute after intravenous administration. This is particularly true in the intestine where the values at this interval are in the order of five to eight percent of the injected dose. With calcium and radium, about 50 percent of the total excretion occurred in the first day although this value is undoubtedly somewhat low because of the lag in fecal elimination. In the case of strontium somewhat less than 30 percent of the total excretion occurred in the first twenty-four hours; however, the decrease in the rate of elimination with time was less than those for calcium and radium. The data are presented in Table 2. The data indicate considerable quantitative differences in the behavior of the three elements. At all intervals the retention of calcium was greater than that of strontium which was in turn greater than that of radium. The total excretion of strontium, while initially less than that of radium, became almost identical to that of radium after 30 days. Furthermore, examination of the partitions of the radioactive materials in the excreta, reveals that about 40 percent of the total excretion of strontium and radium was contained in the urine as compared to a value of eight percent for calcium. This value is not in good agreement with that of Pecher (1942) who, after 36 hours, found 13.7 and 20.4 percent of the injected dose in the urine and feces, respectively, of mice injected intravenously with calcium lactate containing Ca^{45}. There is, however, agreement that the retention of calcium is greater than that of strontium. Furthermore, the experiments of Anthony and coworkers (in press) with strontium and those of Norris and Evans (in press) with radium show the retention of these two materials to be significantly less in mice than in rats.

The rate of elimination of radium in both urine and feces was observed to decrease exponentially with a slope, as plotted (Fig. 1), very close to −1.5 in either case. These data were obtained from four

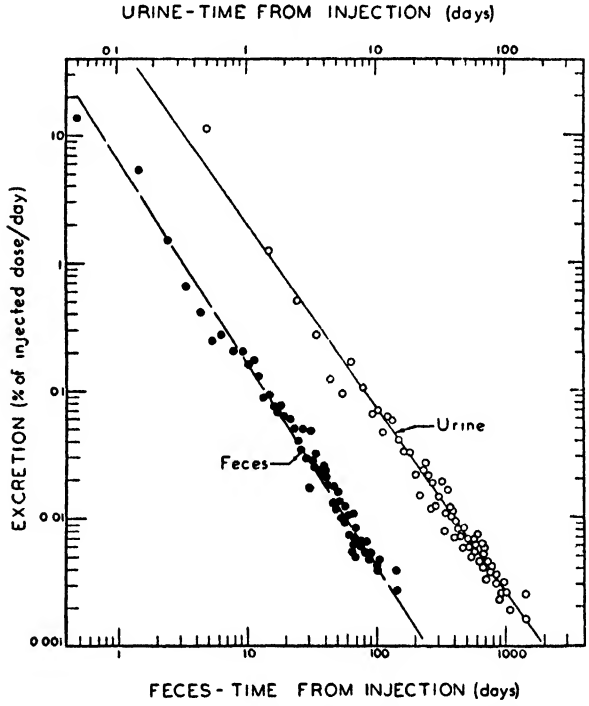

Fig. 1. Excretion of radium injected intraperitoneally into rats.

adult, male Sprague-Dawley rats injected intraperitoneally with 200 μc. of radium each. We have found no significant differences in the rate or quantity of excretion of radium administered either intravenously or intraperitoneally. These data were selected for presentation since they represent the most extensive study of excretion in a single experiment which we have done to date. Equations for

excretion were determined from these data by linear regression of log E (percent injected dose excreted per day) on log T (time in days). They became:

for urine, $E_u = 2.10T^{-1.45}$ (1)
for feces, $E_f = 6.66T^{-1.60}$ (2)
and for total excretion
$E = 8.80T^{-1.56}$ (3)

The percentage of the injected radium excreted in the time interval (t_1, t_2) may be calculated by integration of equation (3). The calculated excretion of radium by these rats between the tenth and three-hundredth days was only 3.7 percent.

Anthony *et al.* (in press) has reported that $Sr^{89, 90}$ injected intraperitoneally into adult, male Sprague-Dawley rats behaves in a fashion similar to that just discussed for radium. The exponential decrease in rate of excretion, plotted on the same scale as for radium, was found by these workers to have a slope of −1.16. The equation for the curve thus becomes

$E = 9.3T^{-1.16}$ (4)

The calculated excretion between the 10th and 300th post-injection days is 17 percent. This agrees well with the data presented in Table 2 which indicate also that the decrease in rate of elimination is less for strontium than for radium.

Blood and Soft Tissues

As might be anticipated, the maximum concentrations in the blood, following the intravenous administration of either calcium, strontium, or radium, were obtained at the earliest examined interval after injection. The values were taken with whole blood in the case of strontium and radium and with serum in the case of calcium. Values for serum calcium were corrected to that of whole blood on the premise that 50 percent of the total volume of blood is serum. The percentages of the injected dose contained in one gram of blood (Fig. 2) at one minute after injection are seen to group around one percent. Some error undoubtedly exists in the values obtained at the early time intervals because of the difficulty in standardizing the injection and the variable times required for bleeding the animals.

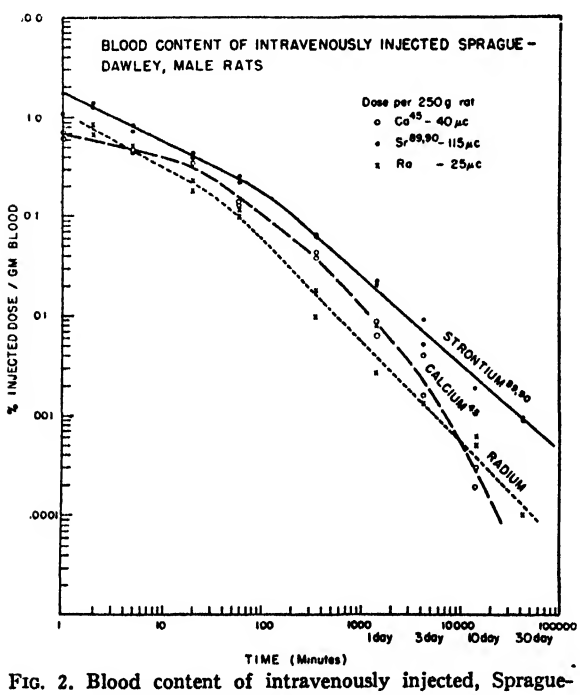

FIG. 2. Blood content of intravenously injected, Sprague-Dawley male rats as a function of time.

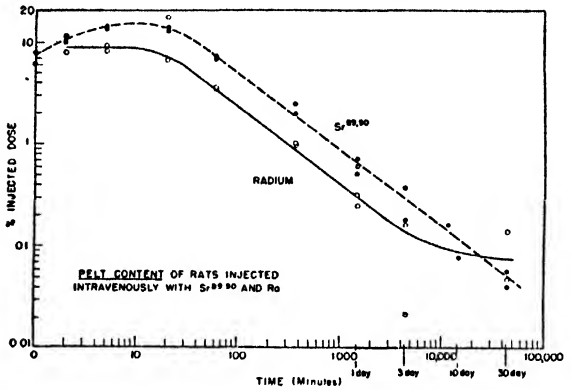

FIG. 3. Pelt content of rats injected intravenously with $Sr^{89, 90}$ or Ra as a function of time.

The slopes of all three curves are observed to change rather sharply at 60 to 100 minutes after injection. It is interesting to observe that this is about the time at which the maximum concentration of these materials is reached in the bones. This point will be discussed in more detail presently. The blood concentration of strontium appears to remain significantly above that of either calcium or radium at all times. Since the decrease in rate of excretion must parallel that of the blood, this again is in accord with the evidence presented earlier to show that the rate of excretion of strontium is maintained above that of radium. With the data at hand it is not possible to state definitely the relative significance of the curves showing the concentrations of blood calcium and radium.

Certain of the soft tissues—namely, the pelt, muscle, liver, kidneys, and intestines—shortly after injection were found to contain large quantities of the administered dose of either calcium, strontium, or radium. The material found in the liver, kidneys, and intestines probably represents largely excretion and the blood content of the organs. The content of the pelt (Fig. 3) for both strontium and radium lies in the vicinity of 10 to 15 percent of the injected dose during the first 20 minutes. Considerations of the concentrations of these materials in blood and the quantity of blood contained in the pelt indicate

at once that a considerable part of the strontium or radium content of the pelt must have been incorporated into its metabolic cycle. Data on the Ca^{45} content of the pelt shortly after injection are incomplete; however, preliminary indications are that its

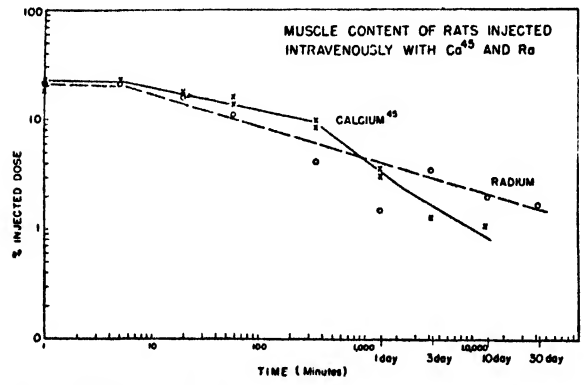

FIG. 4. Muscle content of rats injected intravenously with Ca^{45} or Ra as a function of time.

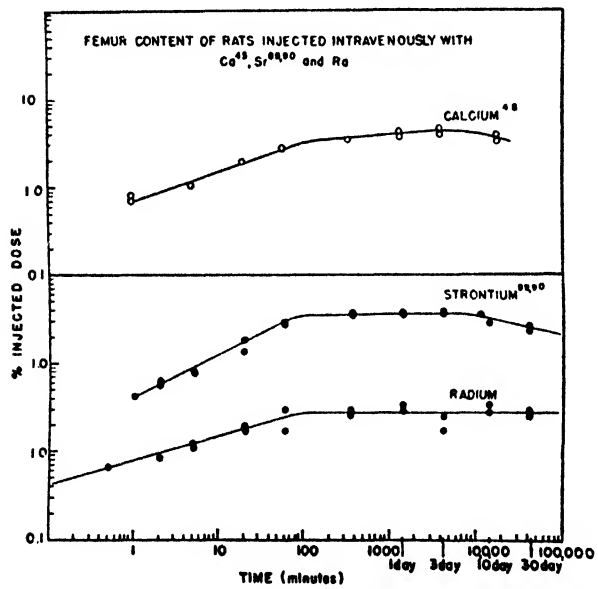

FIG. 5. Femur content of rats injected intravenously with Ca^{45}, $Sr^{89, 90}$ or Ra as a function of time.

behavior is closely similar to that of strontium and radium. Comparison of the rate of decrease in the concentration of the pelt with that of the blood shows that the two are nearly identical. It is thus evident that at least a large portion of the calcium content of the pelt is in a state of rapid turnover and in equilibrium with that of blood.

The content of muscle following the injection of calcium and radium is shown in Figure 4. The same considerations mentioned before show again that a significant portion of the content of the muscle represents calcium or radium which has been actively metabolized. The rate of decrease in concentration of muscle is considerably less than that of blood.

Uptake in Bone

The intravenous injection of the alkaline earths is followed by a rapid uptake of these materials in bone. The bones reached their maximum content in approximately 100 minutes for all three materials and, in the case of the femur (Fig. 5) and scapula (Fig. 6), showed no significant loss for the next six to eight days. With strontium a definite reduction in the content of both the femur and scapula began to

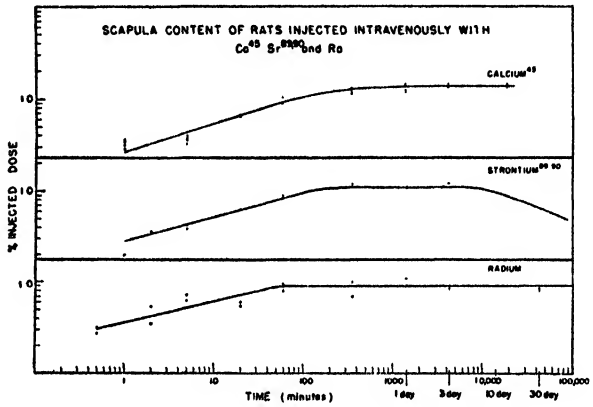

FIG. 6. Scapula content of rats injected intravenously with Ca^{45}, $Sr^{89, 90}$ or Ra as a function of time.

appear after about seven days. In the case of radium no appreciable reduction in the content of either bone was apparent as long as 30 days after the bones had attained their maximum concentration. Similar data for calcium have not been extended far enough to determine what behavior may be expected beyond 10 days. However, there is little indication of any significant decrease in the content of the scapula and femur by 10 days after injection.

Comparison of the maximum values reached by these bones with the three elements shows again that the retention of calcium > strontium > radium. With the scapula, retentions of 1.2 percent, 1.0 percent and 0.85 percent were observed for calcium, strontium and radium, respectively. The femurs gave values of 4.5 percent, 3.5 percent and 2.5 percent of the injected dose for calcium, strontium, and radium, respectively. It is impossible to determine at this time whether the failure to note any significant decrease in bone concentration during the 7 days following the attainment of maximum concentration is due to the existence of a mechanism which requires a definite time interval between the accumulation and excretion of these materials or whether the effect is produced by the redistribution of that fraction of the dose originally deposited in the soft tissues. Discussion of the subject is further

complicated by the well known fact that the minerals of bone do not metabolize as a homogeneous unit.

Those bone salts most recently deposited must also be the ones in most intimate contact with the circulating blood. It would appear, therefore, that the content of these isotopic materials in bone should reach a maximum and immediately begin to diminish as the amount in the blood diminishes. This does not occur. Even if we consider the most unfavorable case—the specific activity of the whole bones as compared with that of blood—we see that the specific activity of whole bone (0.025-0.045 percent injected dose/mg. calcium) at one day is approximately that of blood (0.04-0.15 percent injected dose/mg. calcium) but that after 10 days the specific activity of blood has dropped to 0.01 percent injected dose/mg. calcium or less with the specific activity of bone remaining relatively constant.

This seems to indicate that certain physical and/or metabolic factors are involved which prevent the over-all picture from being that of simple equilibria and exchange as it is ordinarily considered.

Metabolism of Intraperitoneally Injected Radium

The second series of experiments which we should like to present at this time deal exclusively with a study of the metabolism of radium injected intraperitoneally into a large series of adult (240 gm.), male Sprague-Dawley rats. These animals were injected at seven dose levels ranging from 0.91 μc./gm. of animal to 0.02 μc./gm. of animal. All the rats were housed in air-conditioned quarters and maintained on the farm until they became moribund or died. At this time the animals were autopsied and the remains were analyzed for radium content.

As stated previously, the general pattern of uptake, excretion, and retention of these animals, insofar as was observed, was nearly identical to that found following intravenous administration. However, because of the length of time over which the experiment continued and the range of dose levels employed, two unexpected phenomena were observed which appear significant from the standpoint of their effect on the calcium metabolism of the rat.

Retention of Radium

Total-body retention of radium was measured in 100 rats at injection levels ranging from 0.93 to 0.02 μc./gm. The variables investigated were (a) the injected amount, I, expressed in μc./gm., (b) the percent of injected amount retained in the animals, R, and (c) the time in days since injection, T.

As was indicated previously, the percent of injected radium which is excreted in the time interval (t_1, t_2) may be calculated by integrating the curve of rate of total excretion expressed in equation (3). The calculated excretion of radium by these rats between the 10th and 300th days was only 3.7 percent. The calculated excretion between the 10th and 20th days in the rats was 1.4 percent. The mean retention, however, decreased from 57 percent at about 1.0 μc./gm. to 30 percent at 0.02 μc./gm. All these rats survived longer than 10 days. We may, therefore conclude that the retention differences observed in rats at different injection levels are not due to differences in excretion made possible by the variations in length of survival at the different dose levels.

In Figure 7 the retention is plotted against time for all animals, using a separate symbol for each injection level in each of the two experiments. The over-all dependence of percent retention on time, disregarding injection level, is

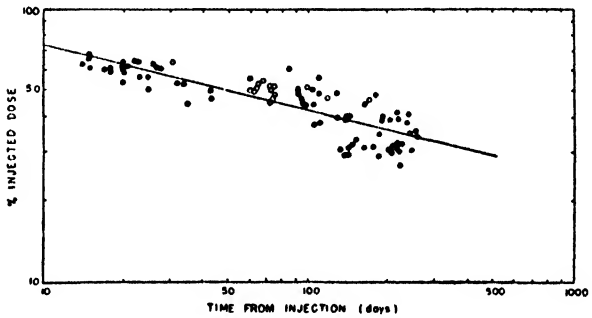

FIG. 7. Retention of intraperitoneally injected radium in rats as a function of time for all dose levels. Separate symbols are used for each dose level.

$$\log R = -0.229 \log T + 2.0842 \qquad (4)$$

As was just indicated, the actual measured excretion is not sufficient to account for this drop in retention with time. If the scatter for each separate group of animals is traced out, it will be seen that the individual groups have very small slopes. This is brought out in Fig. 8, which shows the retention slopes for individual groups. The average of all retention slopes is −0.0407, which should be compared with the over-all retention slope of −0.229, which is derived by disregarding the injection level.

The previous discussion has established that (a) the measured excretion of a given animal amounts to about three percent of the injected amount between the 10th and 300th days after injection, and (b) that retention at individual injection levels after 10 days has only a slight dependence on time.

The relation between retention and amount injected is given graphically in Figure 9 in which the retention of each animal is plotted against injected amount. The mean of each group is indicated by a circle, and the linear dependence of log R on log I is shown by the straight line drawn through the points. The equation of this line was determined by least squares and is

$$\log R = 0.177 \log I + 1.757 \qquad (5)$$

The data presented in this discussion make it clear

that the retention of radium in the rat increases with increase in injected amount, according to equation (5).

Similar data have been gathered and analyzed in the same fashion concerning the retention of radium administered intraperitoneally to both mice

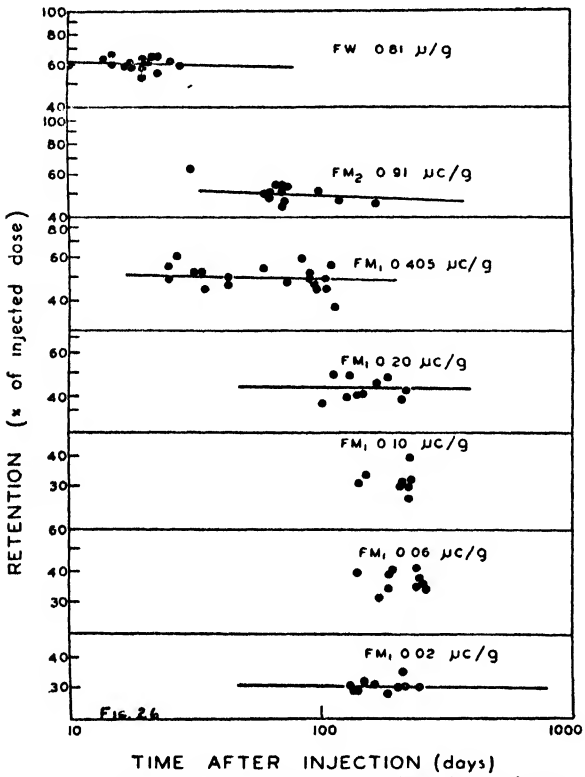

FIG. 8. Series of curves showing the independence of retention of intraperitoneally injected radium on time (after 10 days) at any single dose level.

and rabbits. The dependence of retention on injected amount and on time is the same in both these species as for the rat except for quantitative differences. Both mice and rabbits over the same dose range exhibited considerably greater dependence of retention as a function of dose than did rats.

No good explanations have occurred to the authors for this unexpected behavior. The work of Benjamin (1933), Scholtz (1931) and McLean (1939) showing the formation of colloidal calcium phosphate in the serum, under conditions where the concentration of calcium or phosphate are considerably elevated, must be taken into account. It is possible that the greater insolubility of radium phosphate may be sufficient to favor the colloidal state. According to McLean an increase in the concentration of colloidal calcium phosphate favors metastatic calcification.

Two other possibilities for explanations for this dependence of retention on dose lie (a) in the realm of normal metabolic phenomena in which thresholds and the saturation of certain sensitive systems may be important and (b) in the possibility that this may reflect damage done by the ionizing radiations of the radium to the systems controlling calcium metabolism so that the release of radium from the bone is slowed or stopped altogether. This possibility should be given careful consideration since it may be calculated that the radiation dose produced by 1 μc. of radium in one gram of tissue is close to 420 rep/day. Furthermore, Bloom (in press) has reported that the presence of radium in the bones of mice caused the "development in the metaphysis and the end of the shaft, between three and 40 days after the intraperitoneal injection of 1.0 μc./g., of an atypical dense, fibrous bone."

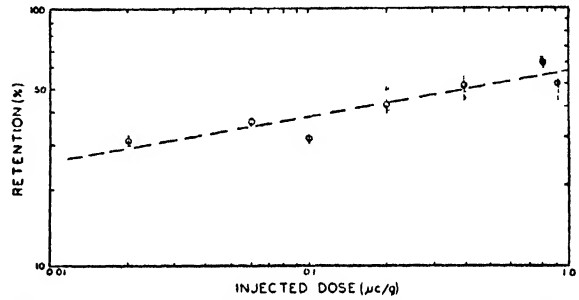

FIG. 9. Plot indicating the dependence of retention of intraperitoneally injected radium in rats on dose level.

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DISCUSSION

NEUMAN On the basis of studies *in vitro* on the fixation of radioisotopes by bone ash (Falkenheim, Neuman and Hodge, 1947; Neuman, Neuman, Main and Mulryan, in press), we have postulated that one of the principle factors involved in the skeletal fixation of radioactive elements *in vivo* is ionic exchange. Ions present in the surfaces of the crystals of the bone mineral substance undergo exchange with similar ions present in the extracellular fluid (Neuman and Riley, 1947; Neuman, Neuman, Main and Mulryan, 1948).

Using the results of studies *in vitro* to interpret data obtained on the intact animal is beset with many hazards. We may logically expect that in the animal variables will be operative quantitatively and even qualitatively altering the experimental situation. Thus, in the case at hand, say, metaphysis and diaphysis, variations in crystal size, crystal growth, relative circulation, the ratio of solid to liquid phase, concentrations of the ions, etc., all may be expected to give variations in isotopic pick-up although the principal physico-chemical event, which we assume to be ionic exchange, may be identical in both areas.

A preliminary attempt to evaluate some of these variables gave interesting results. Bone was carefully dissected from the femurs of young and adult chinchilla rabbits. Three representative areas were taken: metaphyseal, subperiosteal, and diaphyseal bone. By published techniques (Falkenheim, Neuman and Hodge, 1947) the percent of the total phosphate which was available for exchange was determined in each of the three fractions before and after ashing in an ethylene glycol: KOH mixture (Hodge, Crowell, Line). The results are given in Table 1.

TABLE 1

% Exchangeable P

Sample	Ashed	Fresh
Young periosteal bone	12.7	50.4*
Young metaphyseal	13.0	29.3
Adult shaft	13.4	9.2

It is evident that the actively calcifying areas in contrast to compact, non-growing bone, show much more surface exchange and these differences are abolished by ashing.

It would appear, therefore, that the exchange hypothesis, modified to include the physiological factors mentioned above, is compatible with your data and the interesting results of Dr. Darby.

It must be remembered that in areas where actual crystal growth is taking place, the exchanged ions will be trapped *inside* the crystals preventing further equilibration with the extra cellular fluid. Thus in Dr. Darby's rachitic animals Ca^{++}, though it entered the surfaces of the crystals in the epiphysis first, was quickly redistributed throughout the skeleton. In those animals receiving Vitamin D, active crystal growth trapped the exchanged Ca^{++} and redistribution took place quite slowly.

NORRIS: It is, of course, reasonable to assume ionic exchange to be of fundamental importance in the metabolism of bone, and the results of our studies are certainly not incompatible with this idea. Although the final process of fixation may be accomplished through the act of exchange, it must be considered that the process itself is governed strictly by numerous physiological factors. Thus, in your data concerning exchangeable phosphorus in various types of fresh and ashed bone, the differences observed after ashing, if other factors remained constant, could be attributed to the removal of cellular and organic constituents which may contribute considerably more to the overall process than can be accounted for by simple ionic exchange. The possibility also exists, of course, that the change in values may have been effected by corresponding changes in surface areas during ashing.

NOONAN: Do you have any data on calcium or strontium retention showing dependence of retention upon dose administered?

NORRIS: We have made a preliminary attempt to determine whether the retention of strontium is dependent upon the dose level. To date, however, the results are inconclusive. We have not yet attempted to determine whether the retention of calcium is affected by dose level.

THE APPLICATION OF THE ISOTOPE TECHNIQUE TO THE STUDY OF THE METABOLISM OF GLYCINE

D. RITTENBERG

The application of the isotope technique to the problems of biochemistry is still in its infancy. Indeed the major efforts of the investigators in this field have been devoted as much to study of the technique itself as to its application to specific problems. These investigations have outlined the scope of the technique and its limitations. The isotope technique, applied with discretion, has solved questions with which the older procedures had been unable to cope. Of even more importance, it has developed an entirely new class of questions which it seems to be able to solve. It is now scarcely possible to enumerate the experimental successes which this new technique has had. There is one, however, which has profoundly affected our approach to all biochemical problems of the living cell. Almost every experiment dealing with the metabolism of a naturally occurring compound reveals the astonishing rapidity with which a labeled precursor is incorporated into protoplasm and with which the elementary building stones of the complex protoplasmic constituents are synthesized. Most components of the living cell whether large or small are in a state of flux, being rapidly degraded and resynthesized from their precursors (Schoenheimer, 1942; Schoenheimer, Ratner and Rittenberg, 1939).

This ceaseless flow of chemical reactions appears, at first sight, purposeless. From a thermodynamic viewpoint the proteins, fats, and polysaccharides are unstable with respect to hydrolysis to their component amino acids, fatty acids or monosaccharides. These compounds are also thermodynamically unstable in the presence of oxygen. Not only are the hydrolytic and oxidative reactions exergonic, but the living cell is richly endowed with enzymes which catalyse these reactions. For this reason the structural components of the living cell are continuously being degraded and oxidized to the excretory products. To compensate for this chemical erosion, the living cell has available a battery of synthetic reactions which counteract the degradative reactions by rebuilding the protoplasmic structures from the components of the diet and of the cellular debris. The secular stability of the living cell results, not from an absence of chemical reactions but from the exact balancing of the degradative (exergonic) reactions by the synthetic (endergonic) reactions. The living cell maintains its unstable structure by unceasing work. This system though energetically wasteful has certain flexibility which is desirable for a living cell. Since not all the compounds which the organism requires are continuously present in the diet, it must have available mechanisms to synthesize these required compounds from those which are either universally present in the diet or easily derived from dietary components. For example, the absence of stearic acid in the diet can be rectified by synthesis of stearic acid from acetic acid which in turn is derivable from several compounds present in the diet. If this mechanism for the synthesis of stearic acid operated only when stearic acid were needed, a mechanism to start and stop the synthesis would be required. It is apparently simpler for the cell to permit the synthesis to proceed continuously and to maintain a suitable level of stearic acid by balancing the synthetic and degradative reactions.

This complex of reactions is most completely seen with a nutritionally dispensable dietary constituent, for here we can observe, not only the incorporation and removal of the compound from the protoplasmic structures and its degradation to the excretory products, but also its synthesis from its precursors. It was the latter reaction which, prior to the development of the isotope technique, made the investigation of the fate of the nutritionally dispensable dietary constituents so difficult, since it was impossible to deprive the organism of the compound by merely excluding it from the diet.

Our laboratory has for the past ten years been interested in the intermediary metabolism of such a nutritionally dispensable substance, glycine. That glycine is nutritionally dispensable for the rat was well known (Rose, 1938). Its functions, other than that involved in its use for protein synthesis and for the detoxification of benzoic acid, were quite unknown. The complete elucidation of the metabolic fate of glycine would involve the separate study of the two carbon atoms, the nitrogen atom, the two identical oxygen atoms, and the α hydrogen atoms. The hydrogen atoms of the amino group and that attached to the carboxyl group are so weakly bound that they rapidly exchange with the hydrogen atoms of the water molecule. It is thus impossible to associate any particular hydrogen atom with the amino or carboxyl group of any one glycine molecule. A complete study of the metabolism of glycine should thus require five isotopic labels, two for the two carbon atoms, one for the nitrogen, one for the oxygen and one for the hydrogen. Such a complete labeling has not as yet been carried out. Nevertheless the results obtained with N^{15} labeled glycine have thrown a considerable light on the metabolism not only of the amino group, but also of the carbon chain. The mechanisms which remove the labeled

amino group and replace it with unlabeled nitrogen proceed rapidly but not so fast as completely to "wash out" the N^{15}. For this reason it has been possible to trace the fate of the carbon chain of glycine by labeling the compound with N^{15}.

When a small amount of glycine labeled with N^{15} is administered to either a rat or a human the amino acid, after absorption, mixes with free glycine present in the organism. This mixture of labeled and unlabeled glycine can undergo various reactions. Some is directly incorporated into the proteins, a small part is employed for the synthesis of other compounds for which glycine is a precursor and the remainder is oxidized. The ammonia resulting from this degradation is partly employed for the amination of keto acids to form amino acids. The remainder of the nitrogen is excreted as ammonia and urea. To a first approximation whatever is not excreted is deposited as amino acid residues in the proteins. Measurement of the rate of excretion of the

TABLE 1. RATE OF EXCRETION OF THE NITROGEN OF LABELED GLYCINE ADMINISTERED TO A HUMAN SUBJECT

Weight of Subject—64.4 kilograms
Nitrogen Excretion per 24 hours—14 grams

Time hours	Labeled nitrogen excreted Percent of test dose
0- 3	7.3
0- 6	12.3
0- 9	17.5
0-12	22.9
0-18	28.5
0-24	33.0
0-30	36 0
0-36	38.9
0-44	41.5
0-48	42.5
0-60	45.3
0-68	47.3
0-72	48.0

N^{15} of the amino acid thus will reflect the rate of interaction of glycine nitrogen with the cellular proteins. The more slowly the nitrogen of the amino group is excreted the more rapid must be the interactions of the glycine with the proteins.

We have carried out this type of experiment with human subjects. 10 mg. per kilo weight of glycine containing 32 atom percent excess N^{15} in the amino group was administered by mouth in one dose and the total N^{15} excreted in the urine was determined. The data of a typical experiment are given in Table 1. The excretion is rapid for the first 30 hours by which time 36 percent of the administered N^{15} has been excreted. After this time the rate of excretion drops and by the 72nd hour the total N^{15} excretion has risen to but 48 percent. Fifty-two percent of the nitrogen, administered as glycine, is still within the organism. Since the amount of free glycine in the organism is minute it is clear that the remaining glycine nitrogen must have been deposited in the structural elements of the cells. Quantitatively the major nitrogen constituents of the cells are the proteins and it may thus reasonably be deduced that about 50 percent of the dietary glycine has somehow been incorporated into the proteins.

The data from such experiments can be represented analytically by an equation of the form

$$P = 100\,A\,(1 - e^{-Bt}) \qquad (1)$$

where P = percent of test dose excreted
t = time in days
A and B are constants.

A mathematical analysis of this problem indicates that the constant A is related to E, the quantity of nitrogen excreted per day, and S, the quantity of nitrogen converted to tissue proteins per day by the equation

$$A = \frac{E}{S + E} \qquad (2)$$

For the case of the experiment given in Table 1, A is closely equal to 0.5. S and E are therefore equal. The synthetic mechanisms are, in this case, as fast as the excretory ones. Approximately the same relationship between the excretory rate and the rate of protein synthesis exists in rats on a diet containing 15 percent protein.

Such experiments while they qualitatively indicate the rapidity of the interaction of the dietary glycine with the cell proteins cannot easily be interpreted quantitatively. To obtain such information the proteins themselves must be investigated.

Such experiments, on the interaction of dietary glycine with the blood proteins, have been carried out in the human. Glycine labeled with 32 percent N^{15} was administered at a level of 650 mg. per kilo weight per day for two days. Samples of plasma proteins were obtained at intervals and analysed for their N^{15} content. The results of a representative experiment are shown in Figure 1. During the feeding period the isotope concentration of the blood proteins rises rapidly. This is the result of the incorporation of the nitrogen of the labeled glycine into the protein. Not all the nitrogen which enters is still part of a glycine molecule. Indeed during the experiment an extensive redistribution of the amino group occurs. Complete data on this reshuffling is not available for the human but, in similar experiments carried out in rats of the nitrogen incorporated into the liver proteins, only one third is still attached to glycine, while two-thirds has been transferred to other amino acids (Schoenheimer, Ratner and Rittenberg, 1939; Shemin and Rittenberg, 1944). The mechanism of this transfer is unknown. It even is not known whether glycine is directly oxidized to yield ammonia or whether glycine, before oxidation,

must first be converted to some other amino acid.

Since the total quantity of plasma proteins does not increase during the experimental period the incorporation of labeled amino acids must be a replacement reaction, the labeled amino acids replacing identical amino acids present in the protein. Since a replacement of an amino acid requires the rupture and reformation of at least two carbon-nitrogen bonds this process involves the degradation and reformation of a protein molecule. The rate of this reaction cannot be determined from the slope of the rising portion of the curve since the isotopic concentrations of the amino acids available to the cell for synthesis are not known. Even the isotopic concentration of the glycine is unknown for the dietary glycine has been diluted by an unknown amount of glycine resulting from the breakdown of tissue proteins.

On cessation of feeding of the labeled glycine the isotope concentration of the plasma proteins promptly begins to decline. This fall is the result of the same reactions which increased the isotope concentration of the proteins during the period in which the labeled glycine was fed. Now, however, unlabeled glycine from the diet is replacing labeled glycine in the protein. Since the isotope concentrations of the amino acids now available for protein synthesis is very close to zero, an estimate of the rate of protein formation can be made. In a period of about eight days the isotope concentration of the plasma proteins falls to half its initial value. This indicates that in this period about half of the peptide bonds have been ruptured, labeled amino acid residues removed and non-labeled amino acids introduced by the closing of two peptide bonds for each amino acid residue. From the chemical standpoint this process must be described as the degradation and resynthesis of proteins. Half the plasma proteins are degraded and resynthesized in a period of eight days. Similar rates for the turnover of the proteins of the rat liver and the plasma proteins of the rabbit have been found. This value for the regeneration of proteins is a maximum value since we have assumed that the isotope concentration of the amino acid mixture used for the resynthesis of the proteins is very low. This is equivalent to assuming an infinitely rapid mixing of the amino acids liberated from the hydrolysis of the plasma proteins with an infinitely large metabolic pool. If this condition is not met the true regeneration rate will be even more rapid. That the rate of peptide bond formation can be more rapid has been demonstrated for the tripeptide, glutathione (Waelsch and Rittenberg, 1941, 1942). Here the half life time of the structure has been estimated to be about two to four hours. Experiments on the rate of uptake of labeled nitrogen into liver proteins of rats show it to be independent of the dietary conditions. Dietary variations seem to have little effect on the rate of incorporation of N^{15} into the liver protein suggesting that the mixing of the amino acids liberated by the hydrolysis of the proteins with those in the metabolic pool is quite complete.

The relation between turnover rate and fraction synthesized per day is given by equation:

$$k = \frac{\ln 2}{t_{1/2}} = \frac{0.69}{t_{1/2}} \qquad (3)$$

where $t_{1/2}$ is the half life time of the reaction and k is the fraction reformed per day. This reasoning is strictly valid only for the case of a homogenous system, a situation which certainly exists neither for

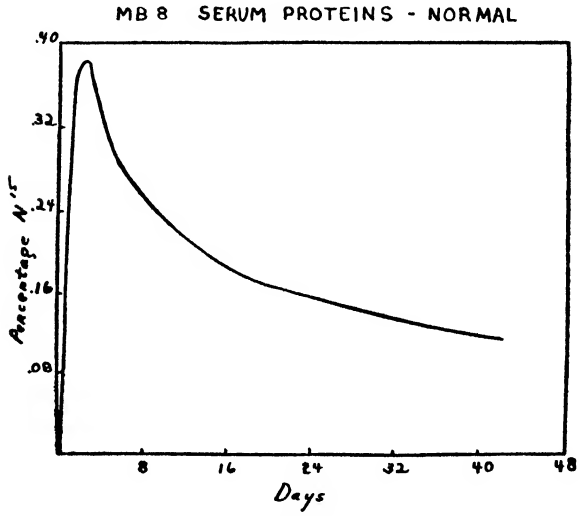


FIG. 1.

the case of the plasma proteins nor the liver proteins. These substances are known to be heterogeneous. Our deductions are thus somewhat unclear since we are only able to give an average turnover rate of the mixture. Strictly speaking such an average is of no theoretical interest but from a practical standpoint it is of greatest value in assisting us in the still obscure field of reaction rates of proteins. In an attempt to reduce the degree of heterogeneity of the plasma proteins we have electrophoretically separated the plasma proteins into the usual fractions. Analyses of these samples shows essentially the same reaction rates for each fraction.

Ever since the chemical lability of the protein in the cell was discovered the question has arisen as to whether the incorporation of the amino acids involves a total breakdown and resynthesis of the protein or individual replacements of amino acids into an otherwise unchanged protein molecule. This question has as yet not received a direct answer. Indirect evidence has been accumulating which is consistent with the "total synthesis" hypothesis. The half time, $t_{1/2}$, for the incorporation of glycine, leucine, tyrosine, lysine, and histidine into rat liver protein have been estimated. These values cluster

about a value of eight days. If the incorporation of amino acids were the result of a total synthesis then the half times for all amino acids should be identical. On the "repair" hypothesis there would not necessarily be any relation between the turnover rates for the different amino acids. The similarity of the values of $t_{1/2}$ for these 5 amino acids may, of course, be fortuitous but at present we incline to the "total resynthesis" hypothesis.

As previously stated, not all of the labeled nitrogen present in a protein after the feeding of labeled glycine is still attached to the carbon chain of glycine. A large amount has been shifted to other amino acids. The mechanisms responsible for this transfer are largely unknown. It may reasonably be guessed that the glycine is oxidatively deaminized to yield ammonia. Indeed, Olsen, Hemingway and Nier (1943) have shown, after administration of carboxyl labeled glycine to rats, that 50 percent of the labeled carbon appears in the respiratory CO_2 within 16 hours. Not only is the amino group removed but the carbon chain of glycine is rapidly oxidized to CO_2. The ammonia so liberated can now be utilized for the amination of keto acids. Investigation of the isotope concentration shows it to be highest in the fed amino acid; the next highest concentration is always found in the glutamic acid. Apparently the reactivity of glutamic acid is greater than that of any other amino acid. In two cases, however, a more direct route for the transfer of the amino group of glycine to another amino acid exists.

After labeled glycine was fed to a human, some of its constituent amino acids were isolated from the plasma proteins. The isotope concentration of the glutamic acid was 0.240 atom percent excess. As might be expected the amide nitrogen, representing the metabolically labile ammonia, had almost the same isotope concentration (0.23 atom percent excess) as the glutamic acid. For comparison the isotope concentrations in the tyrosine, leucine and histidine were 0.060, 0.081, 0.011 atom percent excess respectively. The isotope concentration in the cystine, however, was 0.450 atom percent excess, suggesting that a route, other than that passing through ammonia, exists. While a chemical relationship between glycine and cystine is not immediately apparent, Binkley and du Vigneaud (1942) and Stetten (1942) have shown that serine can be converted into cysteine. The overall relationship is probably glycine $\leftrightarrow$ serine $\rightarrow$ cysteine. In confirmation, we have subjected the protein hydrolysate to a periodate oxidation and analyzed the liberated ammonia (Nicolet and Shinn, 1939). This ammonia is a mixture of nitrogen derived from serine and threonine. Since threonine is an essential amino acid, it is unlikely that its carbon chain can be derived from glycine. Its isotope concentration must therefore either be zero, as is the case for lysine, or quite low, as in the cases of leucine and histidine.

Since only natural threonine is used for growth it will resemble lysine, in which case only the L-isomer is available for growth, rather than leucine, in which the D-isomer can be inverted to the L-isomer and utilized for growth. If then threonine behaves like lysine, its isotope concentration will also be zero. As the concentrations of serine and threonine are approximately equal in human plasma proteins, the actual isotope concentration in the serine should be approximately twice that found in the ammonia after periodate oxidation or 0.53 atom percent excess. This strongly suggests that there is a mechanism which converts glycine to serine.

The mechanism of the deamination of glycine is, as I have previously stated, unknown. Formally one may write the process as taking place in two steps:

$$NH_2 — CH_2 \cdot COOH \rightarrow NH \rightarrow CH \cdot COOH + 2\,H \quad (4)$$

$$NH = CH \cdot COOH + H_2O \rightarrow CHO \cdot COOH + NH_3 \quad (5)$$

In an attempt to study this reaction, we prepared a doubly labeled glycine containing N^{15} in the amino group and deuterium bound to the carbon ($CD_2N^{15}H_2 \cdot COOH$). This compound was fed to rats together with benzoic acid. Hippuric acid isolated from the urine was then analyzed for both D and N^{15}. If reaction (5) were much faster than reaction (4), then every dehydrogenation which removes a deuterium atom would be followed by the loss of the nitrogen atom. Under these conditions the ratio of the isotopic concentrations in the administered glycine and the glycine of the hippuric acid would be identical. In actuality such is not the case. While the N^{15} concentration in the excreted glycine is but one-third that of the fed glycine, the deuterium concentration is but one-twelfth. The deuterium atom is removed at a rate considerably greater than the amino group. While this finding could be explained by postulating that reaction (4) is reversible and much faster than (5) other possibilities exist. Similar results have previously been found by du Vigneaud *et al.* (1939) with the unphysiological amino acid phenyl amino butyric acid. When the L-isomer was fed to rats whose body fluids had been enriched with D_2O the excreted acetyl phenyl amino butyric acid lost only a small portion of its N^{15} but one atom of deuterium entered the α position.

We have prepared an L-leucine in which the amino group was labeled by N^{15} and the α, β and γ carbon atoms by deuterium. Since leucine has a β carbon atom, there exists the possibility not only of dehydrogenation between the α carbon and the nitrogen atom but also of α β dehydrogenation. Indeed the possibility such α β dehydrogenations had earlier been suggested (Bergman, Schmitt, and Miekelye, 1930), and Bergmann and Schleich (1932) had demonstrated the occurrence in tissues of an enzyme capable of hydrolysing dehydropeptides, acyl derivatives of amino acrylic acid. If dehydro-

genation of amino acids resulted in the formation of an amino acrylic acid derivative and subsequent isomerization to an imino compound, deuterium would be lost not only from the α carbon atom but also from the β carbon atom. By appropriate degradations the deuterium concentrations determined for the α, β and γ positions in the above mentioned quadruply labeled leucine. After feeding to rats, leucine was isolated from the muscle proteins and the iosotpe concentrations again measured.

The isotope concentration of the γ deuterium atom was reduced by a factor of 14, and the N^{15} concentration in the amino group by a factor of 40. It seems quite clear that the hydrogen at the γ carbon atom is not involved in the deamination. The reduced deuterium concentration in this position of the isolated leucine must be due to dilution of the fed leucine by the leucine already present in the protein at the start of the experiment. The difference in the dilutions of the γ deuterium atom and the α amino group was caused by the reversible deamination and reamination of the leucine. In this process, the labeled N of the leucine was removed and replaced by normal nitrogen. Thus while the ratio of the deuterium in the γ position to N^{15} in the fed leucine is 1.96, the ratio for the isolated leucine is 4.60. These data suggest that during the period of this experiment about 60 percent of the labeled amino groups had been removed and replaced by normal nitrogen.

The analytical data for the α hydrogen atom reveal a quite different picture. While the ratio of deuterium in the α carbon to N^{15} in the fed leucine was 1.87, the ratio for the isolated leucine was only 0.655. Clearly the labeled α hydrogen atom was being replaced by normal hydrogen atoms at a rate even more rapid than the replacement of the α amino group. As in the case of glycine and phenylaminobutyric acid, the α hydrogen atom is involved in a reaction which can replace it without removing the α amino nitrogen.

The results for the β carbon atom resemble those for the γ carbon atom. Some of the hydrogen attached to the β carbon atom was lost from the carbon chain in comparison to the γ hydrogen, but the loss is quite small and not sufficient to be consistent with an α β dehydrogenation. This loss is probably the result of enolization from the α keto isohexanoic acid which is an intermediate in the deamination-reamination. The investigation of other amino acids will indicate whether, as seems reasonable, the lability of the α hydrogen atom is a general phenomenon and may throw some light on the role of this reaction. It is of interest in this regard that Krogh and Ussing found, after D_2O is administered to animals, that active muscle incorporates more deuterium into the proteins than does inactive muscle (Krogh and Ussing, 1937).

The fact that mammals can synthesize glycine has been known for many years. Glycine might be synthesized by the amination of glyoxylic acid much as glutamic acid can be formed, either by the reductive amination of α ketoglutaric acid or by transamination. On the other hand some amino acids are formed by conversion reactions as for example, the production of tyrosine by the oxidation of phenylalanine. The isotope technique is without doubt the most powerful method for investigating such conversions.

TABLE 2. UTILIZATION OF VARIOUS COMPOUNDS FOR GLYCINE FORMATION 0.35 MM. OF A COMPOUND LABELED WITH N^{15} AND 0.35 MM. OF BENZOIC ACID WERE INJECTED INTRAPERITONEALLY INTO FASTING RATS

Compound administered	N^{15} in isolated hippuric acid* Atom percent excess
Glycine	36.0
Ammonia	0.2
L—Serine	18.2
D—Serine	0.6
L—Glutamic Acid	2.2
D—Glutamic Acid	0.1
DL—Aspartic Acid	1.8
DL—Proline	2.1
L—Alanine	1.1
L—Leucine	0.8
Ethanolamine	0.3

* Calculated on the basis that the compound administered contained 100 atom percent excess N^{15}.

If after the administration of a labeled compound A, the label is subsequently found in compound B, this may be taken as proof that A has been converted to B. The closer the isotope concentration of B approaches that of A, the more direct is the conversion route. Unfortunately, the isotope technique is of no assistance in determining which compounds should be tested as possible precursors. Further, since biochemistry is largely still an empirical science, some other criteria must be employed in the choice of a few of the many possible precursors. In these situations, organic chemistry has on several occasions been shown to be a reliable guide. Dr. Shemin, when he initiated this investigation, was aware that heating serine in alkaline solutions resulted in its decomposition with the production of appreciable quantities of glycine (Daft and Coghill, 1931; Nicolet, 1931). He therefore synthesized and tested as precursors of glycine several amino acids including L-serine (Shemin, 1946). While he could have isolated glycine from the tissues of these animals, he simplified his experimental procedure by taking advantage of the fact that administration of benzoic acid results in the excretion of benzoyl glycine in the urine. By this procedure, the animal can be forced to excrete a sample of glycine into the urine from which its isolation is relatively simple.

The results of this experiment are given in Table 2 (Shemin, 1946). Since the oxidation of any labeled amino acid will give rise to ammonia which may

then be used for the formation of labeled glycine, a control with labeled NH_3 is essential. When labeled NH_3 is administered, the isotope concentration in the excreted hippuric acid is very low; about 1/400 that in the administered NH_3. On the other hand, when labeled glycine is administered the isotope concentration in the excreted hippuric acid is about one-third that of the administered glycine. This dilution is due to mixing of the dietary glycine with the glycine of the metabolic pool. The results after the administration of leucine, glutamic acid, proline,

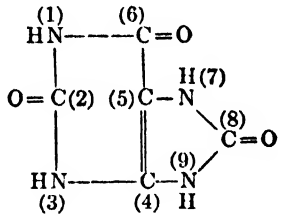


FIG. 2. Uric acid.

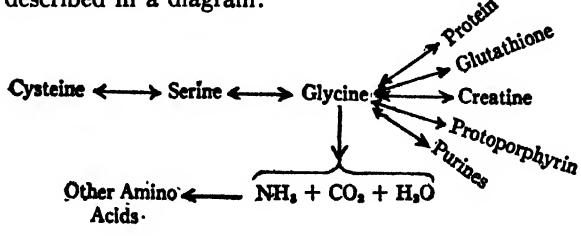
alanine and ethanolamine are all quite low. These values are consistent with the view that in no case is there a direct conversion. With L-serine, however, the isotope concentration in the excreted hippuric acid begins to approach the value obtained after feeding glycine. It is clear that serine is rapidly being converted to glycine. This conversion is specific for the L-isomer. D-serine is almost as inactive as ammonia. The objection has often been raised that such data do not demonstrate the conversion of serine to glycine but merely the utilization of the amino group of serine for glycine synthesis. In theory this is of course correct but actually no example of such a specific transamination is known. In general such data, if used with discretion, give direct information on the fate of the carbon chain. In order to determine which carbon atom of serine is lost in the conversion to glycine, Shemin (1946) prepared serine labeled with C^{13} in the carboxyl group and N^{15} in the amino group. This serine results in the formation of a glycine containing both C^{13} and N^{15}. Indeed, the ratio of C^{13} in the carboxyl group to N^{15} is the same in the isolated glycine and the fed serine, clearly demonstrating that the β carbon atom of serine is lost in the conversion to glycine. These data taken together with those previously given demonstrate that the serine-glycine conversion is biologically reversible.

It is very widely believed that each amino acid has a function or functions other than the mere formation of proteins. The isotope technique has had some of its greatest successes in the discovery of these special functions. When a labeled glycine is administered to an animal, those compounds which are directly derived from glycine will be found to have a high isotope concentration. Shortly after the application of N^{15} to biological problems, Bloch and Schoenheimer (1941) discovered that when isotopic glycine was administered to rats there was formed creatine containing N^{15} in the sarcosine portion of the molecule. Not only did they find the precursor for this portion of the molecule but they further demonstrated that the amidine group was derived from the amidine group of arginine. At the same time du Vigneaud *et al.* (1941) discovered the source of the methyl group of creatine to be the methyl group of methionine. These experimenters had thus completely elucidated the sources of the creative molecule.

The search for other biological derivatives of glycine continued, but lacking any theoretical direction discoveries were largely the result of chance. The next compound to yield to this method of attack was uric acid. Sonne, Buchanan and Delluva (1946) administered carboxyl labeled glycine to pigeons. Isolation of the uric acid showed a relatively high C^{13} concentration to be present in the molecule. Degradation proves that the labeled carbon is in position 4 of the uric acid (see Fig. 2) At the same time they also found position 6 to be derived from CO_2 and positions 2 and 8 from the carboxyl group of acetic acid. In our laboratory we had isolated uric acid after feeding glycine to a human. This uric acid also contained a high N^{15} concentration and on degradation was found to be principally located in the 7 position (Shemin and Rittenberg, 1947). While the relationship of uric acid to the nitrogen metabolism is different in the bird and in man it is apparently synthesized by a similar set of reactions. Glycine is employed for the synthesis, occupying positions 4, 5 and 7 of the uric acid. More recently Abrams, Hammarsten and Shemin (1948) have shown that glycine is employed for the synthesis of guanine in yeast, the glycine nitrogen occupying position 7 as in the case of uric acid.

There is one other biological compound which has been proved to be derived from glycine. Hemin isolated from the red cells of both the rat and the human after feeding labeled glycine contain appreciable concentrations of N^{15}. Experiments have shown that this reaction requires glycine specifically; no other amino acid can substitute for it (Shemin and Rittenberg, 1946). However, when serine is tested, hemin containing a relatively high isotope formation is formed. Dr. Shemin will later demonstrate that the hemin, even in this case, was formed from glycine; the serine first being converted to glycine.

I should like to summarize the data I have today described in a diagram:



When we consider what a wealth of information has been accumulated concerning the metabolism of this single amino acid by judicious application of the isotope technique, we may hope in the not too distant future to have an equivalent amount of information concerning the metabolism of the other amino acids. At that time biochemistry will lose its highly empirical character and become a science on an equal level with chemistry and physics.

I cannot close without expressing my appreciation to my colleagues Dr. Shemin, Sprinson, London, Clarke, and West, without whose labors, both physical and intellectual, little of what I have here reported could have been completed. And, as many of you know, not only I, but all my colleagues are indebted to Mr. Sucher whose skill and patience is largely responsible for the construction and operation of the mass spectrometer in our laboratory.

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MECHANISM OF PHOSPHATE TRANSFER ACROSS CELL MEMBRANES

JACOB SACKS[1]

The presence within the cell of numerous organic phosphate compounds makes the classical approach to the study of membrane permeability inadequate in the case of phosphate ion. Consequently, the standard works on permeability contain little reference to this subject. The use of radioactive phosphorus makes it possible to study the dynamics of the transfer of phosphate across the cell membrane by adequate techniques. The results of such tracer experiments, even on relatively simple systems, have shown that this process is considerably more complex than the transfer of monovalent cations or of chloride.

For example, Kinsey *et al.* (1942) have shown that isotope equilibrium between plasma and the aqueous humor of the eye is rapidly established when tracer sodium or chloride is injected intravenously, but that such equilibrium is much more slowly established following the injection of tracer phosphate. Greenberg *et al.* (1943) have made similar observations in tracer experiments on the establishment of isotope equilibrium between plasma and freshly secreted cerebrospinal fluid. In this case, the P^{32} concentration of the secretion at apparent equilibrium was only a fraction of the plasma concentration.

In the somewhat more complicated system of the red cell suspended in serum, Eisenman *et al.* (1940) found that a rapid transfer of tracer phosphate into the cell took place at 38° C, but the amount of transfer was extremely low at 7° C. Since the difference was much greater than that to be expected from what is a relatively small change in absolute temperature, they concluded that the entry of phosphate into the erythrocyte was the result of enzymatic processes rather than of physical diffusion.

The problem becomes more difficult in the case of tissues such as muscle, heart or liver on account of the necessity of considering separately the intra- and extracellular phases, and the difficulty of effecting such a separation experimentally. Furchgott and Shorr (1943), working on heart slices, and Kalckar, Dehlinger and Mehler (1944), working on muscle and liver, used washing out techniques to remove the P^{32} from the extracellular phase, leaving in the tissue only the intracellular phosphates, organic and inorganic.

Furchgott and Shorr incubated slices of dog heart at 37.5° C in oxygenated Ringer solution containing tracer phosphate, then chilled the material to 2° C, and washed the slices with phosphate-free Ringer solution at this temperature. They found that equal P^{32} concentrations were rapidly reached in the intracellular inorganic P, phosphocreatine (PC), and the terminal phosphate group of adenosine triphosphate (ATP). However, when this intracellular isotope equilibrium had been reached, the specific activities were considerably less than that of the phosphate of the incubating medium. The specific activity of the intracellular P after 30 minutes incubation was about one-fifth that of the P of the external medium. Prolonging the incubation period to 90 minutes increased this ratio only to about one-third.

Kalckar and his co-workers injected the tracer phosphate intravenously, using rabbits for most of the experiments. In the experiments on muscle, the tracer was washed out of the extracellular phase by removing the hind legs of the animal, tying off the vessels to one leg, chilling the preparation in an ice bath, and perfusing the other leg with ice-cold Ringer solution. During the course of the perfusion, most of the PC underwent hydrolysis, and consequently was measured, both chemically and in terms of radioactivity, as part of the inorganic P. The essential finding of the experiment was that the P^{32} concentrations of inorganic P and the terminal phosphate group of ATP became almost equal within a short time after the injection of the tracer. Furchgott and Shorr, and Kalckar, Dehlinger and Mehler concluded from their experiments that inorganic phosphate diffused into the cell, and then transferred radioactive P to the PC and ATP by the reactions of the phosphorylation cycle.

Sacks and Altshuler (1942) studied the time-course of the relative P^{32} concentrations in plasma P and the intracellular P compounds of striated muscle and the heart, in the cat. They concluded that the diffusion hypothesis was inadequate to explain the experimental findings, and ascribed an active part to the cell membrane in the transport of phosphate between the extracellular phase and the interior of the cell. In these experiments the tracer was injected subcutaneously, and a period of one to 24 hours allowed before sampling the tissues. This was done by anesthetizing with pentobarbital, collecting a sample of arterial blood over heparin, and then freezing *in situ*, first the gastrocnemius muscles and then the heart. The P^{32} concentration of the intracellular inorganic P could not be determined directly, but was obtained by calculation. It was

[1] Part of the research work reported here was carried out at Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

assumed for this calculation that the chloride space of these tissues also represented the extracellular phase for phosphate ion, and that the extracellular phase so defined was in equilibrium with plasma water with respect to inorganic P and P^{32}. The figures for chloride space were those determined by Amberson *et al.* (1938) and Yannet and Darrow (1940) for these tissues in this species. The calculation consisted of subtracting from the experimentally determined amounts of inorganic P and its contained P^{32} of the tissues, the amounts that could be presumed to be present in the extracellular phase, thus obtaining the P^{32} concentration of the intracellular inorganic P.

When these calculations were made from the determinations on tissues sampled up to 2 hours after the injection of the tracer, it was found that assigning the entire amount of P^{32} of the inorganic P to the extracellular phase fell short of equality with the concentration of P^{32} in the plasma inorganic P. Evidently the equilibrium of phosphate between plasma and extracellular phase is reached rather slowly. This is in contrast to the rapid equilibration between plasma and extracellular phase which Manery and Bale (1941) and Kaltreider *et al.* (1941) found to take place with respect to Na^{24}, and Manery and Haege (1941) found with respect to Cl^{38}.

In muscle it was found that up to 24 hours after the injection of the tracer, the concentration of P^{32} in the intracellular inorganic P, PC, and the two labile phosphate groups of ATP, remains quite low relative to that in the plasma P (Table 1). Bollman

TABLE 1. TIME COURSE OF P^{32} UPTAKE IN RESTING MUSCLE

Figures denote relative amounts of P^{32} in the various compounds at different times after injection of tracer phosphate. Values are in counts per minute per mg. P, per 10^6 counts per minute injected, per kg. body weight.

Time, hours	Intracellular inorg. P	Phosphocreatine P	Adenosine triphosphate P	Hexose monophos. P	Plasma inorganic P
1	—	26	32	28	13,600
2	—	81	77	31	15,300
4	99	119	85	32	9,675
24	151	133	124	64	843

and Flock (1943) have reported similar findings in the rat, although equivalence of P^{32} concentration between plasma P and muscle PC is attained somewhat more rapidly in the smaller animal.

The uptake of tracer by the PC and ATP of the heart was found to be about 20 times as rapid as in striated muscle (Fig. 1). The data given are for PC and those calculated for intracellular inorganic P. Those for ATP (labile groups) are practically identical with the figures for PC and are omitted from the figure for simplicity. The highest P^{32} concentrations found in this tissue occur at four hours after the injection of the tracer, at a time when the P^{32} concentration in the plasma P has fallen significantly below its maximum value. The intracellular values at this time are only about one-fifth that of the plasma P. From this time on the direction of movement of the P^{32} was reversed, so that at 24 hours lower concentrations are found both intracellularly and in plasma P. But the fall in the P^{32} concentration in the plasma has been so much more rapid that it is now little more than half that

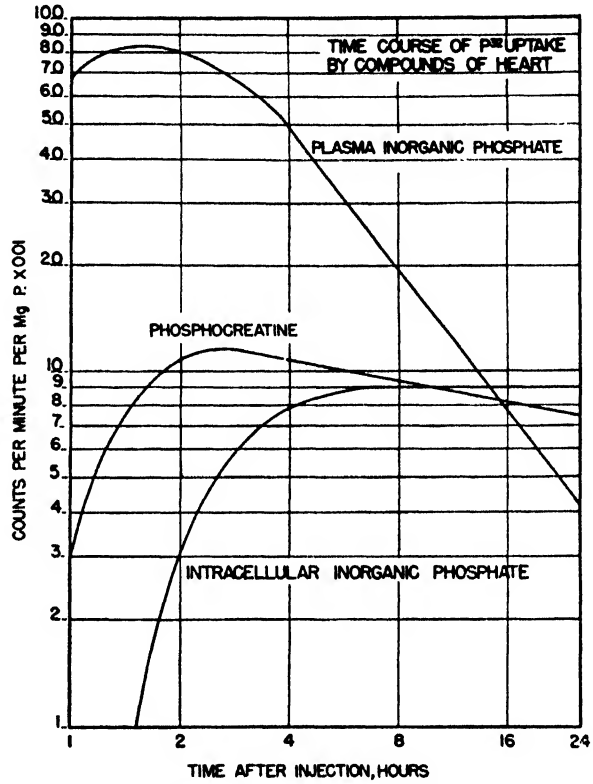

FIG. 1. Time course of P^{32} uptake by phosphocreatine and intracellular inorganic P of heart muscle of cats. Values are counts per minute per mg. P, per 5×10^6 counts per minute injected per kg. body weight.

present in the PC or ATP. Furthermore, the calculated value for the P^{32} concentration of the intracellular inorganic P is somewhat higher than for PC or ATP.

Experiments are now in progress (Sacks, 1948) in which the time-course of the concentrations of P^{32} in plasma and liver phosphate compounds is being studied in the rat. In this case, only ATP and inorganic P come into consideration, since there is doubt that this organ contains any PC. The data obtained to date demonstrate that the rate at which phosphate enters and leaves the liver is very much greater than for the heart (Fig. 2). The peak of P^{32} concentration in the plasma is reached within one

hour after the subcutaneous injection of the tracer, and within another hour the concentration has fallen to about one-third this maximum. In the two- and four-hour experiments, the intracellular P^{32} concentration is higher than that in plasma. In the two-hour experiments, the concentration gradient of

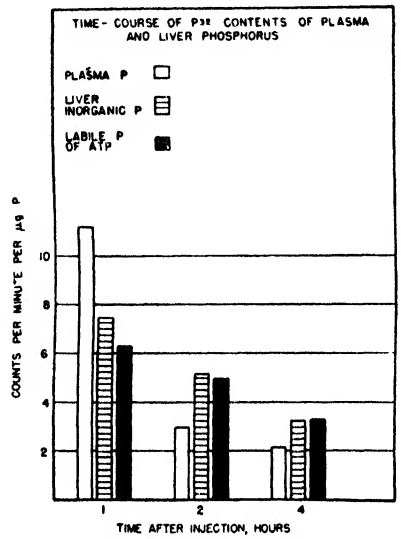

FIG. 2. Time course of P^{32} concentration in inorganic P and labile phosphate groups of ATP in liver of rats. Values are based on 1×10^6 counts per minute injected per kg. body weight.

P^{32} is from intracellular inorganic to ATP to plasma, the same situation as found in the heart 24 hours after injection of the tracer.

These inequalities of P^{32} concentration are difficult to reconcile with the hypothesis that phosphate enters and leaves the cell by simple physical diffusion. Such an hypothesis would require that diffusion take place against a concentration gradient, and would also require that only a fraction of the inorganic P within the cell be available for such diffusion, while the entire amount is able to undergo the interchange reactions which lead to the formation of PC and ATP. This fraction would be about one-half in the liver, one-fifth in the case of heart, and not more than one or two percent in striated muscle.

The experimental data obtained seemed to indicate that the membrane itself takes an active part in the transfer of phosphate into and out of the cell. Since inequalities of P^{32} concentration were observed under conditions of constant P^{31} concentration, it seemed necessary to postulate some chemical process, the velocity of which might vary in different tissues, which took place on the cell membrane. The hypothesis presented (Sacks and Altshuler, 1942) was that the formation of organic P compounds on the membrane from extracellular inorganic P and intracellular organic moieties, served as the means of entry of phosphate into the cell interior. The molecule so formed must be in such a spatial orientation that it is able to penetrate the protein lattice of the membrane. In the case of PC and ATP, the interchanges which are a normal part of the maintenance metabolism of the cell result in redistribution of the P^{32}, so that approximate isotope equilibrium is established between intracellular inorganic P and these two organic P compounds.

It must be emphasized that this attainment of approximate isotope equilibrium within the cell is the consequence of these metabolic interchanges, and is to be anticipated irrespective of how phosphate enters the cell. Under conditions in which the P^{32} concentration of the plasma remains constant, these metabolic interchanges must inevitably lead to equidistribution of the tracer among the several compounds taking part in the interchange reactions. This condition was present in the experiments of Furchgott and Shorr (1943), and they did find such an equidistribution. However, when the P^{32} concentration in the plasma phosphate is changing, there are concentration gradients of the tracer between plasma and intracellular compounds, and the direction of these gradients becomes significant

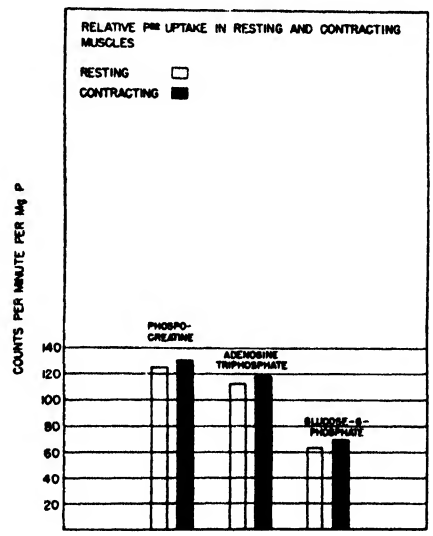

FIG. 3. Relative P^{32} concentrations in P compounds of resting and contracting muscles. Activity consisted of one twitch per second, for 15 minutes immediately before freezing muscles. Freezing two hours after subcutaneous injection of P^{32}.

for the elucidation of the mechanism of entry of phosphate. In the experiments on heart and liver described above, the phase of falling P^{32} concentration in plasma serves to clarify the situation.

The diffusion hypothesis would require that under such conditions, the P^{32} concentration of intracellular inorganic P be intermediate between

those of PC and ATP and plasma. The hypothesis of transfer by compound formation (and breakdown) on the membrane would require that under such non-equilibrium conditions, the intracellular inorganic P have a higher concentration of P^{32} than the organic compound whose formation and breakdown is the means of such transfer. Examination of the data on heart and liver shows that the latter is the case.

In the experiments on muscle (Sacks and Altshuler, 1942), it was found that there were initial differences in the rates of uptake of P^{32} by PC and ATP, but that ultimately these two compounds did come into isotope equilibrium with each other. The hexose monophosphate fraction, however, did not come into such equilibrium even in 24 hours after injection of the tracer. Later experiments (Sacks, 1944a) showed that only the glucose-6-phosphate portion of this fraction fails to come into isotope equilibrium with ATP. Such a finding implies that this compound is formed on the membrane independently of PC or ATP, and also implies that in the muscles of this species, at least, glucose-6-phosphate does not take part in the intracellular inter-

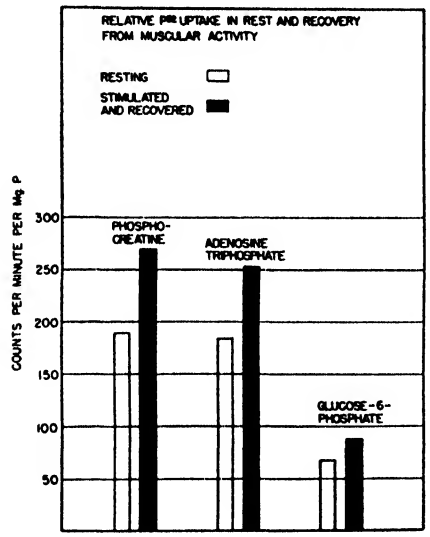

FIG. 4. Relative P^{32} concentrations in resting muscles and those stimulated and allowed to recover. Stimulation one twitch per second for 15 minutes, beginning 105 minutes after injection of P^{32}. Muscles frozen 4 hours after injection of tracer, two hours after end of work period.

change reactions with inorganic P, PC, ATP, and fructose-6-phosphate.

A number of conditions within the physiological range have been studied in which it has been found that the rate of transfer of phosphate across the muscle membrane can be changed, and that these changes in rate are not necessarily the same for all three of the organic P compounds.

Muscular activity has been shown not to cause any significant change in the rate of transfer of phosphate across the membrane. This has been shown in cats (Sacks, 1944b) with respect to all three compounds (Fig. 3), in rats by Bollman and Flock (1943) with respect to PC, and by Flock and Bollman (1944) with respect to ATP. This situation is in marked contrast to the case of potassium. Hahn and Hevesy (1941) and Noonan, Fenn and Haege (1941) have shown with K^{42} that muscular activity may increase several fold the rate of movement of this ion across the muscle cell membrane.

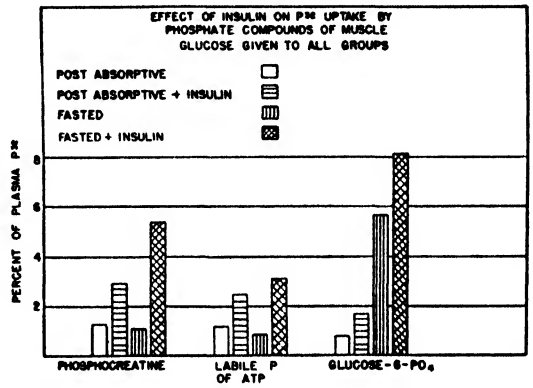

FIG. 5. Effect of insulin on P^{32} uptake by P compounds of muscle in cats. Samples taken four hours after injection of P^{32}, three hours after insulin injection, five units per kg.

Recovery from muscular activity, as distinguished from the activity itself, has been shown to increase markedly the rate of phosphate transfer across the membrane of the muscle cell. This has been shown in cats (Sacks, 1944a) (Fig. 4) and in rats (Bollman and Flock, 1943; Flock and Bollman, 1944). The data of Flock and Bollman (1944) on ATP are of most value with respect to this point, since the rate of contraction was high, and only a relatively short recovery period was allowed, yet the effect of this short recovery period on P^{32} uptake was quite marked.

Finally, the administration of glucose and insulin, particularly to the fasted animal, leads to a great increase in the rate of phosphate transfer across the muscle cell membrane, and that the increase in the rate of transfer is different for PC, ATP, and glucose-6-phosphate (Fig. 5). This effect of insulin was first shown in cats (Sacks, 1945). It has recently been confirmed in the rat, by Goranson, Hamilton, and Haist (1948). These workers also found differentials in the uptake of P^{32} by PC and ATP. They also demonstrated that in animals in shock, the rate of uptake of tracer by these compounds is normal, but insulin given to these animals did not give rise to any increase in P^{32} uptake by these compounds.

In summary it may be stated that the available evidence from the use of P^{32} on the intact animal

indicates that in muscle, heart, and liver, the mechanism by which phosphate is transferred from the extracellular phase to the cell interior is the formation on the cell membrane of an organic phosphate compound which is so oriented spatially that it is capable of penetrating the membrane.

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Discussion

Rothstein: Dr. Sacks' interpretation involves the concept of active participation of the cell membrane in phosphate transport, with the formation and possibly the later degradation thereon, of hexosemonophosphates. Recent work in our laboratory supports the general concept that enzymes are present on the surface of the yeast cell which participate in the metabolism of some external substrates. It seems more than a coincidence that the enzymes which have so far been located on the cell surface include phosphatases, one of which splits the hexosemonophosphates, and also the enzymes which are involved in the initial reactions in glucose metabolism, which are presumably the formation of hexosemonophosphates. The connecting link between metabolism and phosphate transfer in mammalian tissues, as well as in yeast and possibly other micro-organisms involves the presence on the cell surface of enzymes necessary to both processes.

Sacks: Dr. Rothstein is able to prove in yeast what can only be inferred in the mammal, that these enzymes are active on the cell surface. It is gratifying to have this experimental confirmation of such an inference. There is a word of caution needed here: the demonstration of these enzymes on the cell surface does not mean that all the enzyme activity of the intact cell is concentrated on the surface.

There is another point in the data on the mammal in reference to the mechanism of glucose absorption which calls for cautious interpretation. In experiments in which the total dose of phosphate containing the tracer amounted to an appreciable fraction of the inorganic phosphate present in the extracellular phase, there was an early accumulation of P^{32} in the hexosemonophosphate fraction of muscle. At a later period in the experiment, this fraction had a lower specific activity than earlier, and even lower than the ATP. Such a finding can be best interpreted as an accumulation of the HMP on the cell membrane without penetration of this substance into the cell interior. In the experiments on glucose absorption, and when insulin was given, there was no study of the time-course made. Until such data are available, it cannot be stated with certainty that the high specific activity of the HMP during glucose absorption represents the actual entry of HMP into the cell.

THE BIOSYNTHESIS OF PORPHYRINS

DAVID SHEMIN

Although the porphyrins play an important role in intermediary metabolism and very much of their chemistry has been elucidated, mainly by the classical work of Küster, Willstätter, Fischer, Nencki and Piloty, until recently practically nothing was known concerning the biosynthesis of these tetrapyrrole compounds.

In their biosynthesis, the immediate source of the nitrogen atom of the porphyrins may be ammonia or some amino acid which will contribute not only the nitrogen atom but also some or all of its carbon atoms. If the nitrogen source is an amino acid, this need not be one having a cyclic structure. It would appear from recent studies in intermediary metabolism that the biological synthesis of relatively complex molecules in the mammalian organism involves the utilization of relatively simple molecules which appear to be structurally unrelated to the final complex molecule. The concept that simple molecules are involved in the synthesis of the more complex constitutents of the cell represents a significant departure from earlier views on intermediary metabolism. Until very recently molecules were selected as probable precursors for larger molecules merely on the basis of formal similarities of structure. Proline and pyrrolidone carboxylic acid, the anhydride of glutamic acid, are structurally similar to the pyrrole structure of the porphyrins and were regarded by many investigators as probable precursors of the porphyrins. This view is no longer tenable, as will be seen below.

Two simple molecules, acetic acid and glycine, which can be readily synthesized in the animal organism, have been shown to be involved in a variety of biosynthetic reactions. Glycine has been demonstrated to be utilized for the formation of creatine (Bloch and Schoenheimer, 1941), purines (Sonne, Buchanan and Delluva, 1946; Shemin and Rittenberg, 1947) and porphyrins (Shemin and Rittenberg, 1945, 1946a, 1946b). This article will review the evidence for the utilization of glycine for porphyrin synthesis and data on the biosynthesis of the porphyrin, heme.

In order to study the intermediary metabolism of many nitrogen compounds, 66 grams of glycine labeled with N^{15}, the stable isotope of nitrogen, were administered over a period of three days to a human adult and the N^{15} concentrations of many nitrogenous compounds were determined after various intervals of time (Shemin and Rittenberg, 1946b). All the metabolically active constituents analyzed for their N^{15} concentrations reached their maximum value within 24 hours after the termination of the feeding of the isotopic glycine, and thereafter the N^{15} concentrations declined. However, a serial determination of the isotope concentrations of the hemin isolated from the subjects red blood cells revealed the following curve (Fig. 1). An analysis of this curve can demonstrate that glycine is the nitrogenous precursor of heme.

When glycine labeled with N^{15} was fed to the human, the isotope was incorporated into the protoporphyrin during the synthesis of the hemoglobin in the bone marrow. Now, if the hemin were in the dynamic state, that is, if it were continuously being degraded and resynthesized, even though the cell itself were to remain intact, the N^{15} concentration

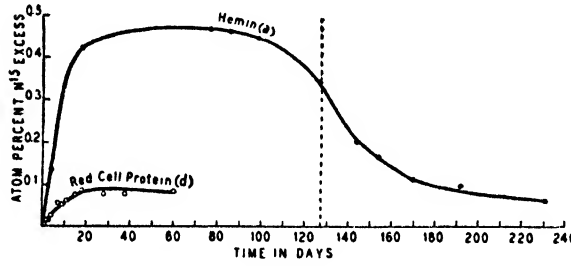


FIG. 1.

would rise to a maximum value during the feeding period and would then decline along an exponential curve. Such a curve was obtained with liver and plasma proteins (Schoenheimer, Ratner, Rittenberg and Heidelberger, 1942; Shemin and Rittenberg, 1944).

If we now look at the curve obtained by plotting the N^{15} concentration of hemin against time we find a thoroughly different type of curve. The curve for hemin (Fig. 1) rises rapidly for about 20 days after the cessation of feeding the labeled glycine, remains at a plateau for the next 70 days, and then falls along an S-shaped curve. Such a curve cannot be the result of a random synthesis and degradation of hemoglobin. Hemoglobin is not involved in a dynamic process or in the flux of synthesis and degradation; the presence of N^{15} in it must be the result of synthesis and incorporation of the hemoglobin during the formation of the red blood cell. These molecules of hemoglobin will then remain with the red cell until the cell disintegrates; in other words, the survival of the labeled hemoglobin will reflect the survival or life span of the red blood cell containing that hemoglobin.

During the 3 day period when labeled glycine was fed, heme containing N^{15} was synthesized in the bone marrow and incorporated into newly formed red blood cells. As these cells reach the peripheral

circulation, they raise the isotope concentration of the heme of the total red cell mass. Up to about the 20th day the curve rises rapidly since the N^{15} concentration of the precursor is comparatively high. About this time, however, the N^{15} concentration in the precursor and in the newly forming heme has fallen so that it is lower than the average N^{15} concentration of the heme in circulation. Nevertheless, the curve continues to rise, though less rapidly, because the new cells, despite a low N^{15} concentration, are replacing old cells which were synthesized before the feeding of labeled glycine and which therefore contain no excess N^{15}. The plateau is reached and maintained when the newly formed heme contains an insignificant N^{15} concentration and is replacing cells formed before the glycine feeding. Eventually, the time arrives when the red cells synthesized during the period in which labeled glycine was fed and which contain heme with the highest isotope concentration begin to be destroyed. As these cells are replaced by new cells with insignificant isotope concentration, the N^{15} concentration in the total heme drops abruptly. This decrease begins about the 80th day. The abrupt decline shown in Figure 1 can occur only because the heme is not significantly, if at all reutilized for new hemoglobin formation. If the heme were reutilized, the isotope concentration would decline very slowly. Precisely such a slow decline is found with radioactive iron which is reutilized when the cell disintegrates (Cruz, Hahn, and Bale, 1941; Hahn, Bale and Balfour, 1941).

These considerations indicate that the heme of the non-nucleated red cell, unlike the constituents of the nucleated cells thus far studied, is not continuously formed and degraded within the cell. They indicate further that red cells are not indiscriminately destroyed but rather their destruction is a function of the age of the cell. From the curve we can calculate the average life span of these cells.

The red cells containing the bulk of the N^{15} and therefore those having the greatest influence on the shape of the curve are synthesized in the early part of the experiment, that is the time when the isotope concentration of the glycine is highest. These cells have an age from one to 20 days and their average age is obtained by taking the mid-point of the upward slope of the curve. This may be taken as the zero point. The declining portion of the curve represents the destruction of the red blood cells containing N^{15}. The maximal point of decline represents the destruction of those cells containing the bulk of the N^{15} namely those cells formed within the first 20 days after the start of the glycine feeding. Consequently, by measuring the time from the mid-point of the rising portion of the curve to the point of maximal decline one can estimate the average life span of the cells. This was found to be 127 days. This value corresponds to the production and destruction of 0.79 percent of red cells per day (Shemin and Rittenberg, 1946b).

With the knowledge of the life span of the red cell, as determined above, it is possible to demonstrate the fact that glycine is the precursor of the protoporphyrin of hemoglobin. Ten days after the start of the experiment the N^{15} concentration of the protoporphyrin was 0.34 atom percent N^{15} excess (Fig. 1). Since the average life span of the red blood cell is about 127 days, approximately 1/13 of the cells are at this time newly formed and contain isotopically labeled heme. The other 12/13 of the cells were synthesized prior to the isotopic glycine feeding and are therefore unlabeled or contain no excess isotope. The newly formed red cells must contain heme having an average N^{15} concentration 13 times as high as the total heme since they are diluted by the cells containing no excess N^{15}. This value is 4.4 percent, that is 13 times the found value of 0.34 percent. Therefore the nitrogenous precursor must also have had this average N^{15} concentration of 4.4 percent during the ten day period. It is clear from quantitative considerations that glycine, the isotopic amino acid fed, is the only compound that could have had this high average N^{15} concentration for the first ten days. On analysis of other nitrogenous compounds such as ammonia and glutamic acid it was found that their N^{15} concentrations did not meet this necessary requirement. The isotope values of these compounds were well below one percent. It would appear therefore that glycine is the nitrogenous precursor of the protoporphyrin of hemoglobin (Shemin and Rittenberg, 1946b).

TABLE 1. N^{15} CONCENTRATION IN HEMIN AFTER FEEDING ISOTOPIC COMPOUNDS TO RATS

Compound Fed	N^{15} conc. in hemin*
	atom percent excess
Glycine	1.4
Ammonium citrate	0.09
Glutamic acid	0.17
Proline	0.16
Leucine	0.07
Acetylglycine	1.3
Serine	1.4

* Calculated on the basis that the compound fed contained 100% N^{15}.

In order further to test the role of glycine in the synthesis of the protoporphyrin of hemoglobin and to eliminate other nitrogenous compound equimolecular quantities of glycine, ammonium citrate, glutamic acid, proline and leucine labeled with N^{15} were administered to rats (Table 1). Proline and glutamic acid were selected since proline and the anhydride of glutamic acid, being structurally similar to the pyrroles, were considered by many investigators as precursors of porphyrins. Leucine was

chosen as a representative amino acid whose intact carbon chain is unlikely to be used for pyrrole synthesis. Ammonia was chosen in order to test the non-specific utilization of nitrogen liberated by deamination of amino acids. It was found that whereas the N^{15} value of the heme after the feeding of glycine was comparatively high (1.4%) the N^{15} concentrations of the hemin after the feeding of proline and glutamic acid, leucine and ammonia was low (0.07– 0.18 atom percent N^{15} excess). The isotope concentrations found in the hemin after the feeding of these latter compounds represent values one would expect to find after the feeding of a non-specific source of isotopic nitrogen which would only enrich the N^{15} concentration of the body nitrogen from which the precursor of heme is synthesized (Table 1) (Shemin and Rittenberg, 1946a).

Subsequently in a similar manner acetylglycine and serine were tested as precursors of heme (Table 1). The finding that acetylglycine functions as glycine confirms the role of glycine as a precursor of the protoporphyrin. A plausible explanation of the utilization of acetylglycine is that this compound is readily hydrolyzed to glycine and acetic acid.

It can be seen from Table 1 that serine functions as a precursor of heme. It has previously been demonstrated, however, that serine is a major source of glycine in the mammalian organism (Shemin, 1946). This conversion comes about by splitting between the α- and β-carbon atoms of serine to yield glycine and a one carbon fragment. Serine may therefore function as a precursor of heme by way of glycine. This experiment does not, however, rule out the possibility that serine is utilized directly for the formation of some pyrrole structure of the porphyrins. Further implications of this finding and the subsequent finding that serine and glycine are mutually interconvertible will be discussed below.

The technique described above for the mammalian red blood cell has also been applied to a study of the life span of the nucleated red blood cell of the bird (Shemin, Hammarsten, Reichard and Arwidson, unpub.). Isotopic glycine was administered to a chicken and hemin isolated from the blood at various periods of time (Fig. 2).

A similar analysis of this curve reveals a life span of the chicken nucleated cell of about 25 days. This value is in good agreement with that found by Hevesy and Ottesen (1945) who kept a chicken at a constant level of radioactive phosphorus and measured the time for the phosphorus of the desoxynucleic acids of the red blood cell to reach the plateau. They found that it required 28 days; the life span of the chicken nucleated red blood cell. This experiment is based on the assumption that the desoxynucleic acids are not in dynamic equilibrium. The finding of 25 days, using N^{15} glycine, should be corrected on the basis of a subsequent finding that nucleated cells are capable of carrying out the synthesis of heme *in vitro* (see below). It seems reasonable to assume that this synthesis must also take place in the peripheral circulation so that the labeled heme will be diluted merely by synthesis of heme from non-isotopic glycine of the diet while the red blood cell is in the circulation. A correction of about ten percent could be applied if the *in vitro* synthesis is a measure of the synthetic rate that may take place in the peripheral circulation. A value of about 28 days will then be obtained and this agreement with the work of Hevesy and Ottesen

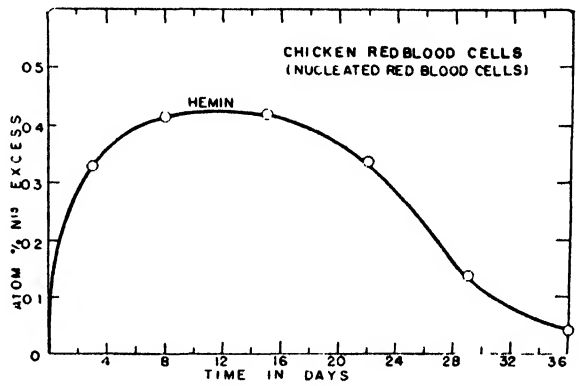


FIG. 2.

(1945) confers more validity on the assumption that the desoxynucleic acids are not in the dynamic state but are synthesized only on formation of a new cell.

In order to facilitate a further study on the mechanism of the formation of heme we sought a simpler biologic system, preferably, for many obvious reasons, an *in vitro* system. In our investigation we have found that blood from patients with sickle cell anemia (London, Shemin and Rittenberg, 1948) and the nucleated red blood cells from the duck (Shemin, London and Rittenberg, 1948) are capable of synthesizing heme *in vitro*. On incubation of these systems with isotopic glycine, heme was synthesized containing isotopic nitrogen (Tables 2 and 3).

In a study of the red blood cell dynamics in sickle cell anemia (London, West, Shemin and Rittenberg, unpub.) similar to that carried out in the normal subject mentioned above, the resulting curve (Fig. 3) is entirely different from that found in the normal (Fig. 1). The curve in Figure 3 can be explained either that the hemoglobin of the red blood cell of sickle cell anemia is in the dynamic state or a complete randomness of destruction exists for the abnormal cell of this disease. We are inclined to hold the latter view, that is that the red blood cell of sickle cell anemia has no definite life span. However in order to investigate this matter, the blood of a subject with sickle cell anemia was incubated with N^{15} glycine and the subsequent isolation of hemin revealed the presence of isotopic nitrogen in

TABLE 2. 200 mg. OF GLYCINE LABELED WITH 32 ATOM PERCENT EXCESS N^{15} WERE INCUBATED AT 37° AEROBICALLY WITH 20 ml. OF HEPARINIZED WHOLE BLOOD

Experiment No.	Hematologic disorder	Reticulocytes	Time of incubation	N^{15} concentration in hemin N
		percent	hrs.	atom percent excess
1	Sickle cell anemia	15	24	0.050
2	Sickle cell anemia	17	24	0.071
3	Sickle cell anemia	20	24	0.071
4	Sickle cell anemia		24	0.077
5	Pernicious anemia*	21	18	0.011
6	Congenital hemolytic jaundice	14	24	0.015
7	Congenital hemolytic jaundice	16	24	0.007
8	Congenital hemolytic jaundice	11	24	0.013
9	Hypochromic anemia		24	0.020
10	Sickle cell trait		24	0.010
11–16	Normal controls		24	0.000–0.015

* 100 mg. of isotopic glycine used.

TABLE 3. 200 mg. OF GLYCINE LABELED WITH 32 ATOM PERCENT EXCESS N^{15} WERE INCUBATED AT 37° AEROBICALLY WITH 20 ml. OF HEPARINIZED DUCK BLOOD AND HEMIN SUBSEQUENTLY ISOLATED

Duck No.	Time of incubation	N^{15} concentration of hemin N
	hrs.	
VP-8	24	0.126*
VP-1	12	0.109
VP-2	18	0.303
VP-3	4	0.051
VP-3	18	0.108
VP-3	24	0.113
VP-4	2	0.032
VP-4	6	0.051
VP-4	12	0.088
VP-9	24	0.090
VP-9	24†	0.006

* This hemin sample was converted to the dimethyl ester of protoporphyrin IX (Grinstein, M., J. biol. Chem. *167:* 515 (1947)). Its N^{15} concentration was 0.124 atom percent excess N^{15}. $C_{36}H_{38}O_4N_4$, calculated N 9.49; found (Dumas) N 9.57.
† Incubated at 5°.

the porphyrin. Thus a synthesis of heme had occurred (Table 2) (London, Shemin and Rittenberg, 1948).

A characteristic of this disease is that a large number of immature red blood cells or reticulocytes occur in the circulation. Therefore the synthesis of heme *in vitro* may be due either to the mature but pathologic cell or the immature red blood cell, the reticulocyte. In an attempt to differentiate the cell responsible for the synthesis, blood of subjects with other blood dyscrasias, also characterized by a high reticulocyte count, was incubated with isotopic glycine and no appreciable synthesis occurred as was found with normal human blood (Table 2). This, however, does not rule out the reticulocytes of sickle cell anemia for these may be qualitatively different. It was subsequently found that blood from another patient with pernicious anemia was capable of synthesizing heme on incubation, and blood from a rabbit containing almost 100 percent reticulocytes, as a result of frequent bleedings, was capable of readily synthesizing heme *in vitro*. On incubation of this rabbit blood with isotopic glycine, the heme isolated contained 0.18 atom percent N^{15} excess (see Table 2 for comparison) (London, Shemin and Rittenberg, unpub.).

As mentioned above, it was found that the heme

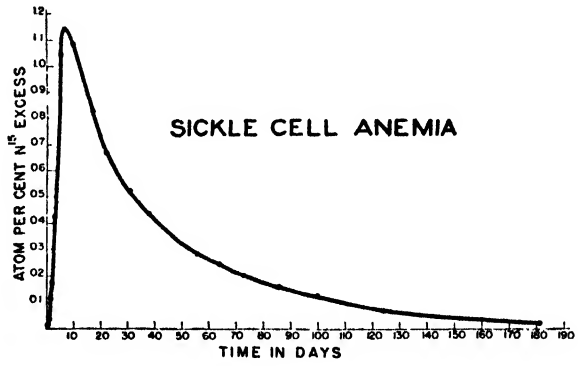


FIG. 3.

of the circulating red blood cell of the mammal is not in the dynamic state and therefore the presence of labeled heme in the red blood cells of the mammal after the feeding of isotopic glycine must come about by formation of heme in the red blood cell while it is still in the bone marrow. The parent cell, in the bone marrow, of the mature circulating red blood cell is nucleated and it is at this stage that heme is laid down in the cell. The circulating red blood cells of the bird are nucleated, in contrast to mammalian red blood cells, and it was thought possible that the erythrocyte of the bird may be

able to synthesize heme *in vitro*. Accordingly, the blood of a duck was incubated with labeled glycine and after some time the heme was isolated as hemin. It was found that the synthesis of heme took place in this *in vitro* system (Table 3) (Shemin, London and Rittenberg, 1948).

Therefore it would appear that both the mammalian reticulocyte and the nucleated red blood cell of the bird are capable of carrying out the involved synthesis of porphyrin *in vitro*. Since the nucleated cell synthesizes heme at a more rapid rate, blood from birds is more readily available and larger amounts can be obtained from many necessary comparative studies, we have undertaken an investigation of the system itself and of the mechanism of heme formation using the nucleated red blood cells of the duck.

It can be seen from Table 3 that heme is readily synthesized from glycine under aerobic conditions and that no synthesis occurs at 0° to 5°.

In Figure 4 are plotted the results of incubating various amounts of N^{15} glycine with 20 ml. of duck blood for 24 hours. It can be seen from the curve that about 25 mgs. of glycine are necessary to saturate the system for a maximum isotopic concentration in the hemin.

In Figure 5 are plotted the results of incubation of 20 ml. of duck blood with 10, 50 and 100 mgs. of isotopic glycine for different lengths of time. It can be seen from the curves that the rate of synthesis is fastest in the first 4 hours and then the rate tapers off but the synthesis still continues for 24 hours.

It has been found that the rate of synthesis in the duck blood system has not as yet been influenced by such additions as iron, glucose, acetic acid, α-ketoglutaric acid and pyridoxal. It has also been found that the rate of synthesis is not changed by storage of the blood for 2 days at 5° prior to incubation with glycine and by decreasing the oxygen tension from 95 percent to 20 percent. Washed red cells alone are capable of synthesizing heme but cells which are broken in a Waring Blender lose their synthetic capacity.

The duck erythrocyte synthesizes not only heme but globin as well. Histidine labeled with N^{15} in the imidazole ring was incubated with duck blood for 24 hours. Histidine isolated from the globin contained an appreciable isotope concentration (0.05 atom percent N^{15} excess), demonstrating the synthesis of peptide bonds.

The duck blood has been also utilized to test other nitrogenous compounds which may be utilized for synthesis of heme. The list of amino acids tested in the rat have been duplicated and extended with negative findings for glutamic acid, aspartic acid, alanine, ammonia and acetylglycine. The behavior of acetylglycine in the duck blood is different from that found in the rat (Table 1). Presumably the duck erythrocytes are incapable of hydrolyzing acetylglycine and this finding rules out the possibility of acetylglycine as an intermediate in the formation of heme.

However, on incubation of duck blood with N^{15} serine, heme was formed containing isotopic nitrogen as was found in the rat (Table 1). This was best

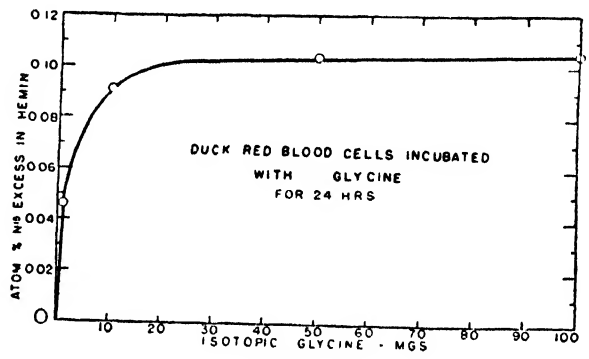


FIG. 4.

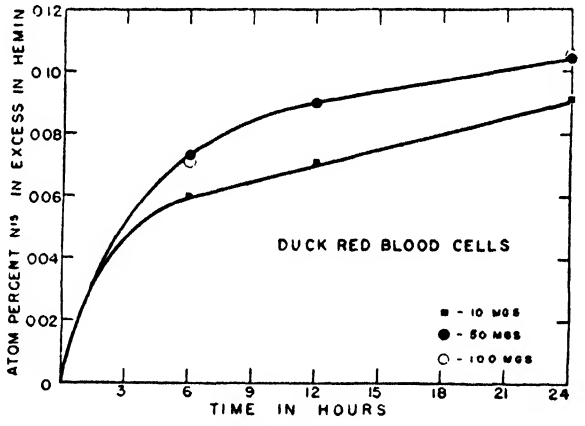


FIG. 5.

explained by the known conversion of serine to glycine (Shemin, 1946). Therefore, it would appear that the duck red blood cell has the enzyme for this conversion. However it was subsequently found, in the human, that the serine to glycine reaction is reversible; not only can serine arise from glycine but it can be converted to glycine (Shemin and Rittenberg, unpub.). At this point one could not decide which of these two amino acids is the immediate percursor of heme. However a definite experiment was set up with duck blood (Shemin, London and Rittenberg, unpub.). A sample of blood was incubated with isotopic glycine and non-isotopic serine and another sample of blood was incubated with isotopic serine and non-isotopic glycine. It can be seen from Table 4 that whereas the non-isotopic serine when incubated along with isotopic glycine hardly affected the N^{15} concentration of the hemin, non-isotopic glycine incubated with isotopic serine com-

pletely abolished the incorporation of isotopic nitrogen in the newly synthesized heme. In another experiment (Table 4) non-isotopic serine on incubation along with isotopic glycine in duck blood did not produce a lower N^{15} concentration in the hemin than when non-isotopic alanine, which is not utilized for heme formation, was added. These experiments demonstrate that while the serine-glycine conversion is reversible and that serine is utilized for heme formation the immediate precursor of the heme is glycine.

Although many other nitrogenous compounds were eliminated as precursors of heme, it was not definitely established that glycine is utilized for the formation of all four pyrrole rings of the porphyrin. Protoporphyrin 9 contains two pyrrole rings (I and II) with methyl and vinyl side chains and two pyr-

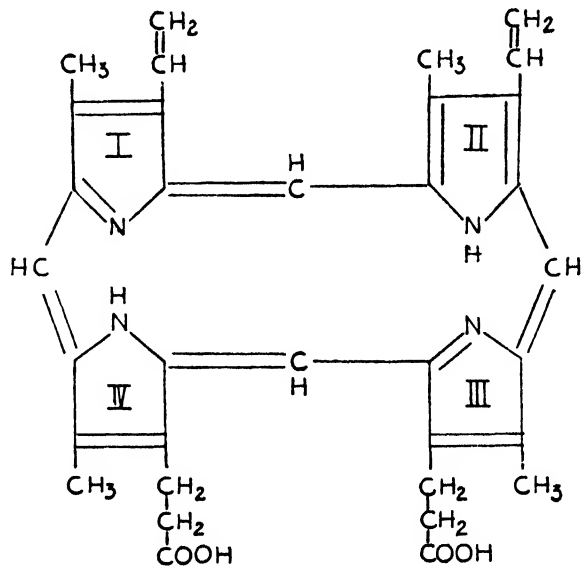
TABLE 4. HEME FORMATION *in vivo* IN DUCK REDBLOOD CELLS

Isotopic compound	Non-Isotopic compound	N^{15} concentration in hemin
Glycine (0.25 mM)	—	0.087
Glycine (0.25 mM)	Serine (1.38 mM)	0.074
Serine (0.25 mM)	—	0.040
Serine (0.25 mM)	Glycine (2.5 mM)	0.005
Glycine (0.25 mM)	Serine (1 mM)	0.133
Glycine (0.25 mM)	Alanine (1 mM)	0.133

role rings with methyl and propionic acid side chains (III and IV) (Fig. 6) and it was conceivable that these two types of pyrrole rings are synthesized in the animal organism in different manners especially if different precursor pyrroles are involved. Degradation studies were undertaken to study the distribution of isotopic nitrogen of N^{15} labeled heme produced by feeding isotopic glycine to a human. The degradation studies were carried out to give unequivocal data in respect to separation and isolation of the two different types of pyrrole rings. The hemin was converted into hematoporphyrin dimethyl ether with an isotope concentration of 0.113 atom percent N^{15} excess (Fig. 7). The hematoporphyrin was then oxidized to yield methyl methoxyethyl maleimide arising from rings I and II and methyl propionic acid maleimide or hematinic acid arising from rings III and IV. The N^{15} concentration of pyrrole rings I and II and pyrrole rings III and IV were the same and equal to that found in the porphyrin (Fig. 7) (Wittenberg and Shemin unpub.). Therefore all the rings of the porphyrin utilize glycine for their synthesis and this finding suggests that the different pyrrole rings in protoporphyrin are derived from a common pyrrole precursor.

The labeling of glycine with N^{15} gives one a direct tool in the study of the utilization of the nitrogen and, unless one postulates special but unlikely transaminating mechanisms, the carbon to which it is attached. This is similar to following the fate of a carbon by labeling it with deuterium. Thus, the described experiments demonstrate that at least the nitrogen and the α-carbon atom of glycine are utilized for the formation of the pyrrole structure of the porphyrin. Indeed, that the α-carbon atom of glycine is utilized in the synthesis of heme has been demonstrated with glycine labeled with C^{14} in the α-carbon (Altman, Casarett, Masters, Noonan, and Salomon, 1948; Radin, Shemin and Rittenberg, unpub.).

Fischer and Fink (1945) demonstrated that glycine and formylacetone condensed to give a product which yielded a positive Ehrlich reaction for pyrroles. From these model experiments it would appear that the α-carbon atom of glycine would be in the α-position in the pyrrole. Though the carboxyl carbon might conceivably become the methine carbon bridging the pyrroles, it has been demonstrated in the dog, rat and duck blood that the feeding or incubation of glycine labeled with C^{14} in the carboxyl group does not result in hemin labeled with C^{14} (Grinstein, Kamen, and Moore, 1948; Radin, Shemin and Rittenberg, unpub.). Therefore, in the



PROTOPORPHYRIN 9

FIG. 6.

formation of the porphyrin utilizing glycine, the carboxyl group of glycine is eliminated. This negative finding does not exclude the possibility that the whole glycine molecule is utilized for the formation of the mono-pyrrole ring but on condensation

of the pyrroles to form the tetrapyrrole structure of the porphyrins, CO_2, in the α-position of the monopyrrole, is split off.

At present very little is known about the actual mechanism of porphyrin formation. As glycine contains but two carbon atoms other compounds must participate with it to form the pyrrole rings. It has previously been shown that the feeding of sodium deuterioacetate to rats results in the formation of deuteriohemin (Bloch and Rittenberg, 1945). This latter finding showed only that some of the carbon atoms of the side chains of heme are derived from acetate, for none of the carbon atoms of the pyrrole rings is bonded to hydrogen. The feeding of compounds labeled with deuterium will therefore furnish only indirect evidence for the participation of these compounds in pyrrole ring formation. However the testing of compounds labeled with an isotope of carbon would yield information in respect to the compounds that participate with glycine in the synthesis of the porphyrin. It has been found that acetic acid labeled with C^{14} in the methyl group, acetic acid labeled with C^{14} in the carboxyl group, pyruvic acid labeled with C^{14} in the carbonyl group enter into the porphyrin while acetone labeled with C^{14} in the carbonyl group, and $C^{14}O_2$ are not utilized (Radin, Shemin, and Rittenberg, unpub.). Together with the α-carbon atom of glycine we can now account for the source of about 22 of the carbon atoms of the porphyrin. The actual position of these compounds in the porphyrin molecule must await the results of the degradations studies which are under way.

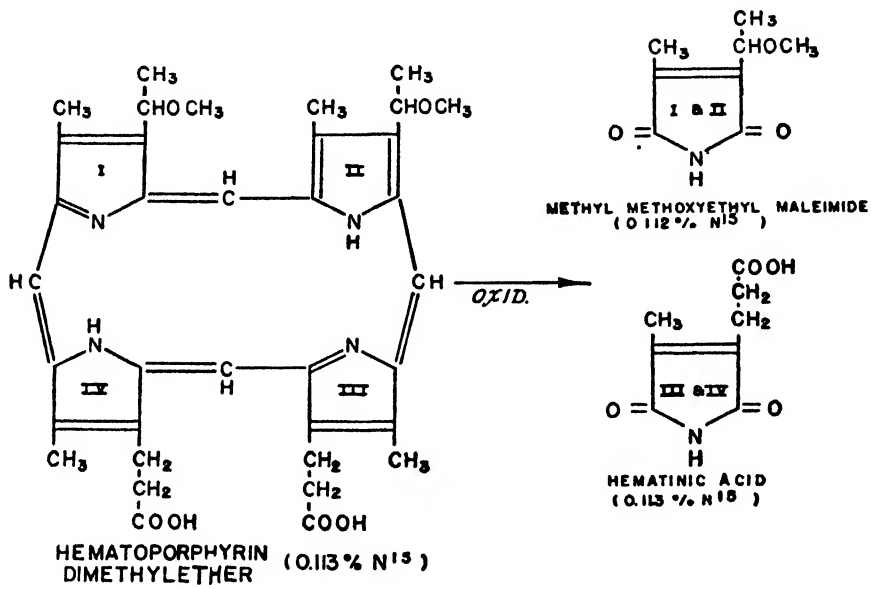


FIG. 7.

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DISCUSSION

NOONAN: Dr. Shemin mentioned the work done by the University of Rochester group using glycine containing C_{14} in its alpha-carbon atom. The work done at Rochester offers confirmation of the value of N_{15} tagging, since high activities were found in hemin isolated from the circulating blood of rats fed labeled glycine. The C_{14} activity per gram of hemin was six to nine times the C_{14} activity per gram of globin and this ratio was maintained in animals rapidly synthesizing hemoglobin.

In connection with the problems of mechanism, I should like to read the conclusions reached by the biochemists in our group. Being a physiologist myself, I am only acting as a transmitting agent for these ideas. The following excerpts are from the discussion section of a paper by Altman, Casarett, Masters, Noonan and Salomon (1948, J. biol. Chem. *176:* 319).

"Shemin and Rittenberg have pointed out that glycine might participate in hemin synthesis in a manner analogous to a reaction recently described by Fischer and Fink. — If the Fischer-Fink reaction is operative in hemin synthesis, it must be assumed that the carboxyl group is split off at some point in the condensation reaction or after the formation of the pyrrol ring.

"It appears more likely that the carboxyl group is removed after the pyrrol ring has been formed, since no enzyme system capable of decarboxylating glycine has so far been discovered, the possible exception being the fermentation of glycine by *Diplococcus glycinophilus.*"

It would appear, therefore, that there is no essential disagreement concerning the views on mechanism of glycine incorporation in hemin as expressed by Dr. Shemin this morning and those developed by Altman *et al.*

THE USE OF TRACERS IN THE STUDY OF ACTIVE ION TRANSPORT ACROSS ANIMAL MEMBRANES

HANS H. USSING

The application of tracers in biological research has revealed a certain permeability to ions (for instance Na, K, Cl) in almost all cells. To explain the deviations from electrolyte equilibrium the assumption of active transport of certain ion species has therefore taken the place of the concept of ion impermeability (*cf.* Krogh, 1946; Conway, 1947).

Qualitatively the concept of active ion transport was supported by the results of tracer experiments; it was very tempting to use tracers also for determining the rate of ion transport.

In several cases the tracer method has already given interesting information concerning active transport processes (see for instance Katzin, 1940; Barker Jørgensen, Leir and Ussing, 1946; Levi and Ussing, 1948; Ilolm-Jensen, 1948). The method was strongly advocated by Krogh, 1946.

In the determination of active ion transport, however, the tracer method involves certain limitations. Before entering into a discussion of our experiments on active ion transport, I consider it useful briefly to account for what tracer experiments actually can tell us.

If we have two NaCl solutions separated by a membrane and we add, say, Na^{24} to one of the solutions, we can determine the rate at which Na^{24} enters the other solution; from this figure and from the net change of the amount of Na in one of the solutions we can calculate the *flux*, that is, the total amount of Na ($Na^{23} + Na^{24}$) which per unit time crosses a unit area of the membrane. Let us denote the flux in one direction by influx and that in the other direction by outflux of sodium.

The information we gain by determining the flux is of a quite neutral nature. If, for instance, the influx is negligible compared with the outflux we may be justified in identifying the outflux with an active extrusion. If, on the other hand, outflux and influx are of the same magnitude several possibilities present themselves. This case is of more than theoretical interest. In many cell types the active ion transport results principally in the maintenance of a steady state. In muscle, for example, the Na-concentration is always lower than in the extracellular fluid. Depending on the (unknown) permeability of the cell membrane, this low concentration may require an active Na-extrusion of any magnitude lower than the total Na-outflux.

Different Types of Ion Exchange

Influx and outflux of an ion can be equal due to simple diffusion, namely when the ions in question are present at the same electrochemical potential on both sides. In muscle fibres, for instance, K^+ is present at higher concentration than in the extracellular fluid; this difference seems, however, to be balanced by an electric potential, the inside of the fibres being negative relative to the outside. According to Boyle and Conway (1941), the K distribution between fibre and surroundings represents a Donnan equilibrium. The K-flux from the low outside concentration to the high concentration in the inside will require no more active transport than the K-outflux does.

But even if the ions have not the same electrochemical activity on both sides of the membrane we may, at least theoretically, find outflux equal to influx without any active transport.

Such a hypothetical exchange system can be simply visualized as a Na-impermeable monolayer containing scattered anions of a substance which forms a stable complex with Na.

Due to thermal movements these complex molecules will sometimes come into contact with the outside medium and sometimes with the inside medium. If the inside solution contains, say, Na^{24} ions these may exchange with Na^{23} in some of the complex molecules and when later these molecules touch the outside solution, Na^{24} will leave the complex in exchange for Na^{23}.

In its ideal form such a mechanism will always take up one Na ion when it gives off another so that no net change in the Na-concentration on either side of the membrane need take place. Such a diffusion we call *exchange diffusion.*

Until now we have assumed that the complex forming anion (B^-) forms a complex with Na^+ only. In fact, it will form at least one more uncharged complex, namely the corresponding acid, BH and, therefore, the foregoing considerations are strictly valid only in a region where the Na-concentration is very high compared with the H^+ ion concentration.

Generally we have, however,

$$\frac{[BH]}{[B^-]} \cdot K_H = [H^+] \text{ and } \frac{[BNa]}{[B^-]} \cdot K_{Na} = [Na^+],$$

where K_H is the acid dissociation constant and K_{Na} the instability constant of the complex BNa.

When combining these equations we get

$$\frac{[BNa]}{[BH]} = \frac{[Na^+]}{[H^+]} \cdot \frac{K_H}{K_{Na}} = \frac{[Na^+]}{[H^+]} \cdot K.$$

(The expressions in the brackets denote thermodynamic activities.) This means that if pH is the same on both sides of the membrane more BH will be formed on that side where the Na-concentration is

lower than on the other side and, therefore, Na will leak out from the more concentrated side in exchange for H^+ ions. But even if such leakage is going on we may find the proportion between Na-influx and outflux much nearer to unity than was to be expected from the electrochemical activities of Na on the two sides of the membrane.

A study of the exchange of Na across the cell membrane of striated muscle has led Dr. Levi and myself (Ussing, 1947; Levi and Ussing, 1948) to the assumption that an exchange diffusion system as outlined above might be responsible for at least part of the outflux of Na from the fibres. It can easily be shown that free diffusion-out is negligible compared with the diffusion-in, since the former process proceeds against the concentration gradient as well as against the electric potential gradient. Active transport may be responsible, or rather must be responsible, for part of the outflux; but a calculation shows that so much Na leaves the fibres that at least half the energy from the resting metabolism of the fibres would be required for its active extrusion. Therefore it is most likely, although not certain, that a considerable part of the Na-outflux is actually due to exchange diffusion. It would be extremely interesting to find some living cell where the magnitude of the flux against the electrochemical potential was definitely so high as to exclude with certainty the active transport as solely responsible factor. Until then exchange diffusion is only hypothetical, but has nevertheless to be considered a possibility. It will thus be seen that the tracer method may give too high values for the active transport. This may happen as a result of exchange diffusion but may also be due to simple diffusion when a concentration difference across a membrane is balanced by a potential difference.

Under other conditions, however, the tracer method will give too low results for the active transport.

Let us consider the following system:

The cell C is separated from the outside medium by the wall A and from the inside by the wall B. We assume ideal mixing in C as well as in O and in I. We further assume the system to be in a steady state with respect to Na^+ so that the Na^+-concentration in each phase is constant. This is brought about by the active transfer of Na^+ across B, so that the flux in both directions across B is b. Through A the flux in both directions due to the diffusion is a. For the sake of simplicity, the walls are taken to have unit area. Now, it is the flux from O to I which can be measured by means of the tracer method (and vice versa). If we wish to relate this quantity to the values a and b it can be done in the following way. Ions originating from I are denoted by Na^+_i (and those from O by Na^+_o). Because b moles of Na^+_i enter C per unit time the steady state demands that b moles of Na^+_i should also disappear.

This Na will leave partly through A and partly through B in proportion to the flux from the cell in the two directions.

Hence, out of the b moles entering C from I in unit time $b \cdot \frac{a}{a+b}$, moles of Na^+_i leave through A and $b \cdot \frac{b}{a+b}$ go back through B.

The flux from O to I (and from I to O) is thus $\frac{a \cdot b}{a+b}$. Only if a is very large compared with b will the flux from O to I be a measure of the active transport through B. In all other cases the flux between O and I is smaller than the active transport. As soon as, in an organ performing active transport, more than one membrane in the way of the ions offers a high resistance to diffusion we shall find the flux across the organ smaller than the true transport. Measurements performed by means of the tracer method do not include what may be called internal diffusion losses. These considerations are of course valid, *mutatis mutandis*, even if net uptake or net loss takes place. At first sight the situation now looks rather discouraging; the tracer method may yield results which are much lower or much higher than the true active transport. Nevertheless, when the results are treated with due caution, the tracer method is an invaluable tool in the study of active transport and I shall attempt to demonstrate this by taking as an example our study of the active salt transport through the isolated frog skin.

Ion Transport Through Isolated Frog Skin

In 1935, Huf found that the isolated frog skin transports chloride ions from the outside to the inside when both sides are bathed with Ringer solution. Two years later, Krogh (1937) showed that living frogs in need of salt may even take up salt (NaCl) from a 1/100 millimolar solution. Since then a similar ability to take up salt has been found in skin, gills or separate organs of a large number of fresh-water animals; but it still seems that the isolated frog skin is the best object for a more detailed study of the salt transport. Without special precautions the skin can be kept alive for nearly 24 hours, the preparation is simple, and it is easy to determine the exposed area.

I shall first show you the apparatus used (Fig. 1). A suitable piece (ca. 3 × 3 cm.) of abdominal skin of a frog is placed as a membrane between the ground edges of two cylindrical celluloid cups. Each cup fits in a flat depression in an ebonite plate and these plates can be pressed together between two centered screws.

The solutions in the two chambers thus formed on either side of the skin are circulated by blowing in air through the side tube A. The solutions then ascend to the funnels F through the tubes B and

return to the chambers through the tubes C. From the funnels samples can be drawn. The funnels are further used for making contacts between the solutions and two calomel electrodes E. The contacts are made through the capillary tubes G. One end dips in the funnel and the other in a small tube H filled with saturated KCl. For the potential measurements a tube potentiometer was used.

The isotopes, Na^{24} and Cl^{38}, were added through one of the funnels, for instance on the outside, and from the concentration and the rate at which the Na or Cl activity appeared in the other chamber, the influx of the ion in question was calculated. In the figures and tables the flux is expressed in μ moles/cm^2/hour.

Let us first consider the influx and outflux of Na^+ as a function of outside Na-concentration.

Figure 2 shows two experiments where the inside solution is Ringer and the outside NaCl concentration is increased in steps from 1 mmol to 115 mmol. The influx increases too but is relatively lower at high than at low concentration.

In Figure 3 the influx of Na^+ is given as a function of outside concentration using data from 20 different

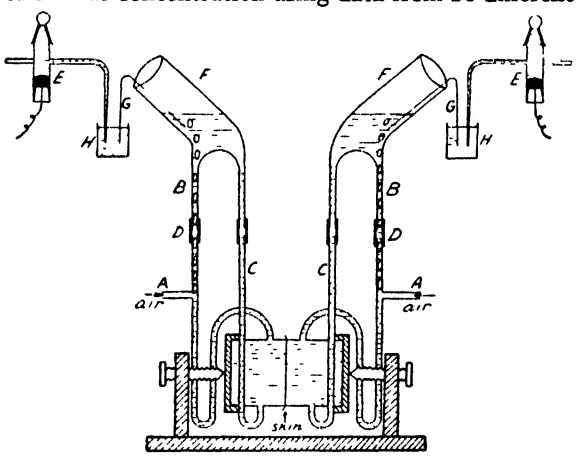

FIG. 1. Apparatus used for the determination of the in- and outflux of ions through the isolated frog skin (see text).

experiments. In all experiments the inside pH was 8.3 and the outside pH near the neutral point (as will be shown later the inside pH exerts a very pronounced influence on the influx of Na, whereas the outside pH seems to have no decisive effect between 5 and 8). The figure shows a considerable scattering of the observations, but the general trend is obviously the same as in Figure 2. The figure shows moreover a number of observations of outflux of Na. These observations were made under conditions as far as possible identical to those prevailing during the influx determinations. The outflux too shows an increase with increasing outside concentration. What is still more important is that in the concentration interval studied the outflux is always considerably lower than the influx. On the basis of Huf's *loc. cit.* results it could be assumed that a net uptake of sodium would take place when both sides of the skin are in contact with Ringer; Katzin, *loc. cit.*, using radioactive Na^+, showed definitely that there is a net transfer inwards of Na when both sides of the isolated skin are in contact with identical solutions containing NaCl and KCl; the present experiments indicate, however, that a net uptake of Na^+ is to be expected even from a millimolar solution.

As already mentioned, the pH of the solutions is of great importance for the flux values. Let us first consider the case where the shifts in pH are brought about by changing the CO_2-tension in the

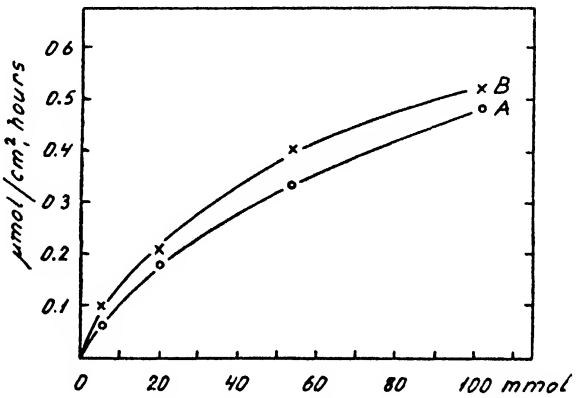

FIG. 2. The Na^+-influx through the isolated frog skin as a function of outside NaCl-concentration. The NaCl-concentration is raised in steps by addition of solid NaCl. A and B denote two separate experiments.

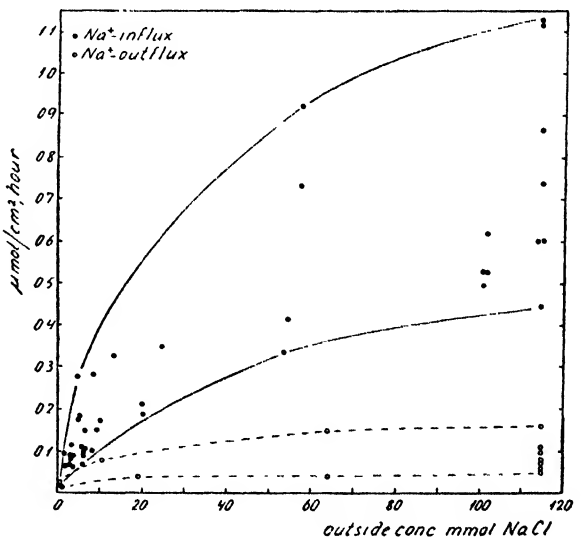

FIG. 3. The Na^+-influx (·) and the Na^+-outflux (o) through the isolated frog skin. Data collected from 20 different experiments for influx and from 6 for outflux. Inside pH in all cases 8.1-8.3.

air used for mixing. Table 1 shows such an experiment, where the CO_2-tension was changed from 12.4 mm. Hg. (1½ percent CO_2) to that of atmospheric air and back again.

The influx of Na^+ and Cl^- was determined in one hour periods. It is seen that the Na-influx is about 3 times higher at the low CO_2-tension than at the amounts of Na-phosphate-buffer. Table 2 shows that even here the Na-influx follows the changes in pH, so that high pH gives a high Na-influx, whereas low pH gives a lower influx. It is evident, however, that the adjustment of the influx to a new pH value takes much longer time (more than one hour) when phosphate is used to shift the inside pH.

TABLE 1. INFLUENCE OF pH ON THE INFLUX OF SODIUM AND CHLORIDE IONS THROUGH THE ISOLATED FROG SKIN. EACH EXPERIMENT COMPRISES THREE SUCCESSIVE ONE HOUR PERIODS. THE pH CHANGES ARE BROUGHT ABOUT BY CHANGING THE CO_2-TENSION OF THE WHOLE SYSTEM

	Concentration mmol.		pH		Na passing in	Cl passing in
	inside	outside	inside	outside	μ mol./hour, cm.²	μ mol./hour, cm.²
	115	2.87	7.1	5.8	0.022	0.012
I	115	2.87	8.3	7.1	0.073	0.014
	115	2.87	7.1	5.8	0.027	0.009
	115	8.04	7.1	5.7	0.030	0.031
II	115	8.04	8.3	6.9	0.098	0.116
	115	8.04	7.1	5.7	0.023	0.008
	115	3.32	7.2	5.7	0.025	0.006
III	115	3.32	8.3	7.1	0.097	0.012
	115	3.32	7.2	5.7	0.064	0 011
	115	10.00	7.2	5.45	0.059	0.007
IV	115	10.00	8.3	6.87	0.170	0.025
	115	10.00	7.2	5.45	0.094	0.013

TABLE 2. THE INFLUENCE OF INSIDE pH ON THE Na^+-INFLUX. THE pH CHANGES ARE BROUGHT ABOUT BY THE ADDITION OF PHOSPHATE BUFFERS

	Period number (one hour each)	NaCl-conc. (mmol) inside	outside	pH inside	outside	Na^+-influx μ mol./cm.², hour	P.D. mV sign refers to inside
	1′	115	1.65	6.72	6.5	0.018	− 8
I	2′	115	1.65	6.72	6.5	0.010	—
	3′	115	1.65	7.77	6.5	0.060	+14
	4′	115	1.65	7.77	6.5	0.061	+19.5
	1′	115	1.64	7.77	6.5	0.095	—
II	2′	115	1.64	7.77	6.5	0.050	—
	3′	115	1.64	6.72	6.5	0.039	—
	4′	115	1.64	6.72	6.5	0.015	—
	1′	115	6.60	8.1	6.3	0.318	+82
III	2′	115	6.60	8.1	6.3	0.276	—
	3′	115	6.60	7.2	6.3	0.161	+51
	4′	115	6.60	7.2	6.3	0.070	+38

high one. Other experiments have shown that the relative change in Na-influx following a shift in CO_2-tension is but little dependent on the Na-concentration. It should be mentioned here that the Cl^- influx shows variations resembling those found for Na^+; but, with very few exceptions, the Cl-influx is much lower than the simultaneous Na-influx.

In another series of experiments the pH of the inside solution alone was changed by means of small

If, on the other hand, the inside pH is kept constant and the outside pH is changed the picture is quite different (see Table 3). The Na-influx is hardly influenced by the shift of pH from 6.6 to 5.25. When, however, the outside pH is lowered to 3.3 the Na^+-influx is nearly stopped. This experiment together with others indicates that somewhere between pH 5.25 and 4.15 the Na-influx begins to fall off rapidly. At the same time the Cl-influx rises.

This probably means that the pace setting factor for the Na-influx (and thus for the Na-transport) is the Na-permeability of the surface layer of the outside. According to Amberson (1936) this layer has the isoelectric point 5.1 in good agreement with the value obtained by means of the tracer method. At higher pH the surface layer is predominantly permeable to cations and at lower pH to anions.

Table 3. The Influence of Outside pH on the Influx of Na^+ and Cl^-. The Outside pH is Lowered in Steps by the Addition of Phosphoric Acid

Period number (one hour each)	NaCl-conc. mmol. inside	NaCl-conc. mmol. outside	pH inside	pH outside	Na^+-influx μ mol./cm.^{2}, hour	Cl^--influx μ mol./cm.^{2}, hour
1′	115	6.36	8.0	6.6	0.105	0.083
2′	115	6.36	8.0	5.25	0.097	0.055
3′	115	5.44	8.0	3.3	0.012	0.175

As to the outflux of Na it may suffice to say that it generally is higher at high pH values. But as the outflux is lower, and often much lower than the influx, the changes in influx actually determine the variations in net transport.

Na-influx and Potential Difference

The Na-influx and the potential difference across the skin seem to be very intimately interrelated. According to Meyer and Bernfeld (1946) the potential difference increases in a characteristic manner with the outside NaCl concentration, so that a higher NaCl-concentration makes the inside more positive relative to the outside. The change in P.D. takes place in a way which recalls the change of the Na^+-influx with outside concentration.

Meyer and Bernfeld further found that the P.D. was closely correlated with the inside pH, so that in typical experiments an increase in one pH unit made the inside solution about 58 mV more positive relative to the outside, as if the frog skin were a glass electrode.

As we have already seen, the Na-influx also follows the inside pH, giving high values at high pH, and vice versa. A similarity in the behavior of P.D. and Na-influx may also be seen in the fact that both change much more rapidly when the pH shift is brought about by changing the CO_2-tension than when phosphate is used. No doubt, the reason is that CO_2 diffuses readily across living membranes in contrast to the phosphate ions.

Briefly, we can summarize: All factors that make the inside solution more positive relative to the outside will also increase the influx of sodium ions.

As already mentioned, the Cl^- influx as a rule goes parallel to the Na-influx although on a lower level. If we compare 24 corresponding determinations of Na and Cl-influx, we see that the Na-influx is much higher than the Cl-influx in 20 cases, about equally high in two cases, and lower in two cases.

Huf (*loc. cit.*), using in his experiments Ringer on both sides of the skin, found that the chloride transport was blocked by cyanide and that, at the same time, the potential difference fell to 0. It may be of interest therefore to consider how cyanide acts on the Na-influx if the outside NaCl concentration is much lower than the inside concentration which, in our experiments, is that of frog Ringer (Fig. 4). Here the outside salt concentration is 3.8 mmoles/1 whereas the inside solution is Ringer as usual. At the arrow, NaCN is added to the inside

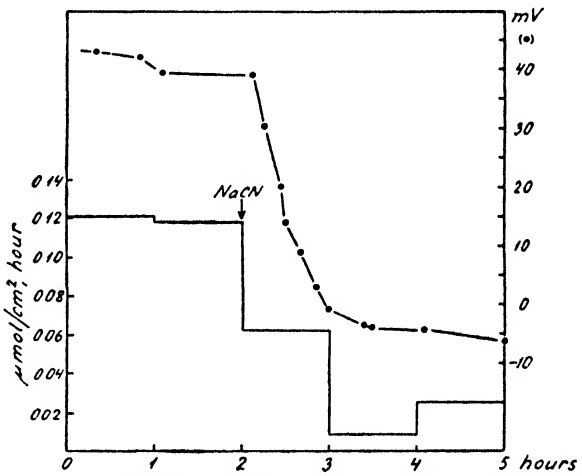

Fig. 4. Influence of cyanide (2 mmolar) on the Na^+-influx through and the potential difference across the isolated frog skin.

solution in an amount sufficient to make the solution 2 mmolar as to CN^-. It is seen that both Na-influx and potential difference decrease. In fact, the potential changes sign, the inside becoming slightly negative relative to the outside. The Na-influx decreases to a small fraction of its original value, but later it rises somewhat again, probably due to post mortem changes in the skin.

The Na-outflux is not reduced by cyanide poisoning and there is in fact a considerable leakage of salt through the skin after CN^- application.

What do these experiments tell us about the nature of the active salt transport?

The Cl^- Transport

Let us take the Cl^- ions in the first place. The inside solution is almost always positive relative to that outside. Qualitatively, it is therefore possible that the Cl^- ions are carried in due to electric attraction. Whether electric forces suffice to bring about a net transport of Cl can be calculated from the Cl^--concentrations and the potential difference across the skin. In order just to keep the Cl from leaking out, the Cl^- ions must have the same electrochemical potential on both sides. If the outside solution is 5 mnormal and that on the inside 115 mnormal as to Cl, the equilibrium requires the potential difference $58 \cdot \log \frac{115}{5} = 80$ mV, a quite reasonable value. If the P.D. is higher a net transport should be assumed to occur. That the P.D. is the motor force for the Cl^- ion may be concluded from the fact that the variations in Cl^- influx are closely correlated with the P.D.

As already mentioned, the Cl-influx as a rule is lower than the Na-influx and, thus, sometimes it may be found that there is a net Na^+ uptake and a Cl^- loss; and in almost all cases some of the Na-ions must enter the inside solution together with some anions other than Cl, just as the outside medium must receive some cations to replace the Na-ions taken up in excess. The most logical assumption is possibly that the Na-ions are in part accompanied by bicarbonate in the inside solution, whereas hydrogen ions enter the outside solution to replace some of the Na-ions.

The Na^+ Transport

Whereas the Cl-ions may thus have approximately the same electrochemical potential on both sides of the skin, this is definitely not the case with the Na^+-ions. The Na^+-ions have to travel in against an electric potential gradient and in many cases also against a concentration gradient. The influx due to free diffusion is therefore bound to be very small indeed compared with the outflux. Nevertheless, the influx has been found to exceed the outflux in all cases. In most experiments the excess is quite considerable. When both sides of the skin are bathed with Ringer the mean outflux is only about 10 percent of the mean influx. It thus seems tempting simply to identify the Na-influx with the active Na-transport.

If, however, we wish to obtain a measure of the transport work done the influx will tend to give too low values because, as mentioned earlier, the internal diffusion losses cannot be measured by means of the tracer method. If, on the other hand, by the rate of active transport we understand the amount of ions actually carried across the skin per unit time the influx will probably give the best approximation to the right value. The internal diffusion losses in this case should be regarded as an inherent part of the transport mechanism. Thus, it becomes a matter of definition whether the Na-influx should be identified with the Na-transport.

The Site of Na-transport in the Frog Skin

It may be useful now to look at the microscopic structure of the frog skin. As is well known, the skin consists of two layers, namely the mesodermal chorion and the ectodermal epithelial layer. The chorion is built up of connective tissue containing blood vessels and smooth muscle cells. The whole structure is a meshwork presenting no serious obstacles to diffusion. Active transport cannot take place here.

The epithelial layer consists of only two or three layers of cells. Nearest to the chorion we find the stratum germinativum consisting of cylindrical cells. Outside the stratum germinativum we have cells some of which resemble normal epithelial cells whereas others are being keratinized. On the outside is the layer of fully keratinized cells.

From a histological point of view the active transport must evidently be ascribed to the stratum germinativum; however, it may perhaps be possible to locate the active transport still more precisely. We know that pH changes in the inside solution result in conspicuous changes of Na-influx as well as of P.D. On the other hand, the outside pH may be varied within rather wide limits before influencing Na-influx and P.D. Thus, the site of transport can be readily reached by diffusion from within, but not from without.

Similarly, adrenaline has no influence at all on salt permeability, Na-influx and potential when added to the outside medium, whereas it has a drastic effect on all three phenomena when it is added on the inside (Barker Jørgensen, 1947; Ussing, 1948.

As the chorion can be left out of the discussion pH change and adrenaline must be assumed to act on that surface of the stratum germinativum cells which is nearest to the chorion.

If this view is correct, the cells of the stratum germinativum extrude Na-ions through the basal cell membrane. As is well known, most animal cells have a Na-concentration which is lower than that of the outside medium and they must therefore constantly extrude Na^+. The difference between the epithelial cells and other cells, therefore, is not that they extrude Na^+, but that they do so only on the side turning inwards. This is certainly a specialization most useful for the animal, since a symmetrical extrusion of Na^+ from the epithelial cells would lead to the rapid loss of all the NaCl in the animal.

The Relation between Na^+-transport, Potential Difference and pH

How is this active Na-transport brought about? Of the details nothing definite can be said; but so

FIG. 5. Cross section of the skin of the frog (*Rana temporaria*).

much is certain: sodium cannot travel the whole way from a lower to a higher electrochemical potential as free ions. Across the membrane where the transport takes place the sodium is bound to permeate in chemical combination, possibly in the form of an uncharged complex. On their arrival at the other side of the membrane the Na^+-ions are set free by some chemical reaction. The complex forming anion may undergo a chemical change which diminishes the affinity to Na (oxidation, reduction, etc.) or some other cation formed during the metabolism may replace the Na-ions in the complex.

For the following considerations it is insignificant in which way the Na^+ is set free from the unknown complex. The only presupposition is that a necessary step in the active transport of Na^+ is the formation of a complex BNa in which form Na^+ is supposed to cross the membrane.

Let us now discuss the origin of the potential measured across the frog skin. From the above considerations it seems likely that the positive charge of the inside relative to the outside is due to the active Na-transport. As Na^+-ions are transferred more rapidly across the skin than the Cl-ions can follow, the inside will be positively charged. This view would explain an important observation made by Huf (1935). As already mentioned, Huf has observed that frog skin in contact with Ringer on both sides transports chloride from the outside to the inside. This transport could be stopped by poisoning with monobromoacetate which also lowers the potential. Most remarkable was, however, the observation that addition of lactate to the monobromoacetate-poisoned skin partly restored both the potential and the power of chloride transport.

We have now seen that also factors like outside salt concentration and inside pH affect potential difference and Na^+-influx in a uniform way. All these observations are in agreement with the assumption that the P.D. is due to the Na^+-transport. But the adoption of this hypothesis has certain interesting implications. How, for instance, can we explain the change in potential following a change in pH of the inside solution? As already mentioned, the inside of the skin behaves like a glass electrode so that the change in P.D. corresponds, at least approximately, to the change in electrochemical potential of the hydrogen ion. How can it be that the P.D. which, according to our hypothesis, is due to Na^+-transport gives a direct measure of changes in pH?

The most reasonable explanation is that the skin (or rather the proximal cell membrane of the stratum germinativum cells) is in fact acting like a glass electrode (*cf.* Meyer and Bernfeld, 1946) being much more permeable to H^+-ions than to other ions.

In analogy with the glass electrode the change in potential across such a membrane will be a measure of the change in H^+-ion activity under one condition, *viz.* that the pH of the cytoplasm of the transporting cells is kept constant.

Thus, we arrive at the conclusion that the Na^+-transport proceeds at a rate exactly necessary to keep the pH of the cells constant. (It ought to be mentioned here that the P.D. response on pH change is sometimes less than the theoretical expectation, especially when the frogs have been starving for a long time. This may mean that the cells are unable to prevent a drift of the protoplasm pH in the direction of the pH applied on the inside of the skin.)

The question may be put whether active Na-transport is at all suited for causing a regulation of the pH of the cells. The answer may be given that active transport of Na^+ means in fact a forced exchange of Na^+ against H^+.

The regulation of the transport in such a way as to fix the cellular pH might of course be vaguely described as biological regulation. It may well be, however, that this apparent regulation is purely automatic. As already mentioned, we must assume the formation of a Na-complex to be a necessary step in the active transfer of Na^+ across the cell membrane. If the amount of Na-complex present on the cellular side of the membrane is the pace setting factor of the transport system, then the pH of the cell interior is bound to have a decisive influence on the amount of complex formed. For any such complex we have, irrespective of its chemical structure,

$$\frac{[BNa]}{[BH]} = \frac{[Na^+]}{[H^+]} \cdot \frac{K_H}{K_{Na}} \quad (cf.\ \text{p. } 193).$$

If for a moment the Na^+ concentration is considered constant we have

$$\frac{[BH]}{[BNa]} = \frac{H^+}{K},$$

where K is a constant.

This formula shows clearly that a decrease in H^+-ion concentration in the cell increases the amount of [BNa]. A higher [BNa] value in turn will tend to increase the active extrusion of Na with the result that the cellular H^+-ion concentration rises. In other words, an automatic pH regulation is established.

As we have seen, the Na^+-influx and the potential also show a parallel dependence on outside NaCl-concentration. This can be easily understood if we assume that the Na^+ extruded from the cells is replenished by diffusion from outside. Then, it becomes clear that the rate at which Na^+ is allowed to enter the transporting cells is a very important factor in determining the rate of active transport and thus the potential difference produced.

As already mentioned, most animal cells seem to be able to perform active Na^+-transport and it is not unlikely that this transport is a general link, though hardly the only one, in the regulation of cellular pH.

But now I am leaving the relatively solid ground of experimental evidence.

In the study of active transport in cells, where outflux and influx of the ions studied are equal, the

use of tracers meets with many pitfalls. Diffusion, electric attraction, exchange diffusion and active transport may contribute to the flux measured, and only a detailed study may reveal the actual phenomena.

Our remarks on the limitation of the tracer method in the study of active transport are of course equally valid for permeability studies. Not only the flux of an ion should be determined, but both the electric potential difference and the concentration difference should be known if a tracer experiment is expected to give other than empirical values.

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Discussion

MICHAELIS: The term "complex compound" plays an important role in this paper. I wish to comment upon the meaning of this concept, which has been used somewhat vaguely on various occasions during the whole history not only of biochemistry but of chemistry in general. A "complex" may mean a salt-like compound which does not, or not appreciably, dissociate into its ionic components. On other occasions, a "complex" may mean a coordinate compound of the Werner type, a central metal ion being combined with a number of "ligands" in excess of the number represented by the "valency" of the metal. Here, it is not necessarily implied that the bond of the central metal atom to the ligands should be always strong and not inclined to reversible dissociation. So, in the ferrocyanide ion, the bonds are strong; however, in a complex, *e.g.* the cobaltous hexammine, the bond is so loose that this complex exists only in the presence of a large excess of ammonia. Neither of these two types of complex compounds fulfills the conditions implied in Dr. Ussing's problems. A "sodium complex," such as postulated here, may mean one of two things: either an organic compound in which Na would play the role of Zn in the compound $Zn(CH_3)_2$. Such organic metal compounds exist only in the absence of water and cannot be seriously considered here. Or, alternatively, and the only thing that may be termed a "complex" worthy of consideration here, is this. Suppose the membrane contains, or consists of, high-polymers such as cellulose, which contains acidic side chains. A good model is the "dried collodion membrane" described by me 20 years ago. Its property of being relatively impermeable to anions must be attributed to the negative charge of the wall of its pores. Sollner has shown recently that this negative charge originates from the fact that, due to oxidation, there are some carboxyl groups present. Then, there is an acidic substance, of which the anions are firmly fixed whereas the cations belonging to it, such as H^+, or Na^+, though movable of their own account, cannot move away from the fixed anions. One kind of cation may be replaced by another as in a Permutite, however some cation must always stick to the negatively charged substance of the membrane. Only in this sense, something that may be called a "sodium complex compound" may be imagined to exist in a membrane.

USSING: Very little can of course be said concerning the chemical nature of the Na-complex which is involved in the active salt transport in the frog skin. I should like to point out, however, that it is not just the question of Na^+ acting as counter ion to some high molecular acid. Krogh found that frogs in need of salt take up Na^+ from a very dilute solution, whereas they never take up K^+. From a mixture of Na^+ and K^+ they are able to remove only Na^+. Though the complex may be salt-like the anion involved must have a high and specific affinity for Na^+, perhaps due to forces similar to those which make some Na^+ salts nearly insoluble in water.

THE SYNTHESIS OF LIVER GLYCOGEN IN THE RAT AS AN INDICATOR OF INTERMEDIARY METABOLISM

HARLAND G. WOOD

The author first wishes to acknowledge his coworkers Dr. Nathan Lifson, Dr. Victor Lorber and Dr. Warwick Sakami who have had a major part in these investigations.

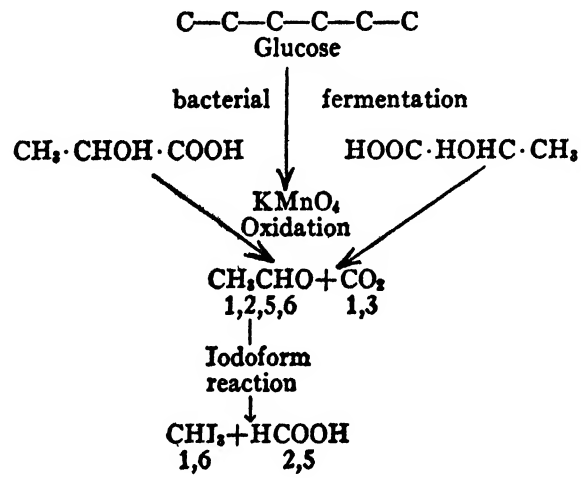
The work to be reported is in part currently under investigation and certain results have not attained the point of completion where one may truly feel he has reached the "talking stage." Nevertheless, it seems worthwhile to present some of the incomplete results; perhaps their shortcomings will generate more discussion than would a completed fully established investigation. At any rate it must be recognized today that the biochemical currents move so fast that one laboratory rarely does a complete job on a subject unattended by others; these results then are but one part of a story which ultimately will be woven from the work of many laboratories.

Introduction

Liver glycogen has long served as a useful biochemical indicator of metabolism; it is traditional to determine whether or not a compound gives rise to carbohydrate precursors by determining whether or not it is glycogenic. It is interesting that through the use of isotopes, liver glycogen is proving to be an even more important biochemical indicator. It has been possible to establish, when certain compounds are fed, that they give rise to characteristic distribution patterns of the isotope in the carbon chain of the glucose unit of the glycogen. By comparing the pattern from known carbohydrate precursors with those from other less clearly understood metabolites it is possible to predict something about the intermediates which may be formed from the compounds whose metabolism is largely unknown.

Glycogen is particularly well suited for this type of study. It can easily be depleted by fasting the animal and when a glycogenic compound is fed the glycogen is rapidly replenished. Because of this, even in as short a time as 2½ hrs. after the feeding of an isotopic compound, the glycogen becomes labeled and in addition because the glycogen is freshly laid down, it forms a fair sample of the metabolic pool of carbohydrate metabolism. The isotope content of the glycogen is thus measurable even when materials of fairly low isotopic content are fed. Furthermore, since the feeding is of short duration not much material is necessary for an experiment.

An additional advantage of the use of glycogen for sampling the metabolic events of carbohydrate metabolism is that isolation of glycogen in relatively pure form is not difficult (Stetten and Boxer, 1944) and also the degradation of the glucose is quite readily accomplished (Wood *et al.*, 1945). One method of degradation which has proved to be very convenient involves the use of bacteria. The sugar is fermented to lactic acid in practically quantitative yield by using a suspension of *Lactobacillus casei*. This lactic acid is in turn oxidized with permanganate to acetaldehyde and CO_2 using the familiar Friedemann and Kendall (1929) method which is designed for the quantitative determination of lactic acid. The CO_2 thus formed comes from the carboxyl group of the lactic acid. Since in the glycolytic cleavage of glucose two lactic acid molecules are formed and the carboxyls of the lactic acid are formed from the 3 and 4 positions of the glucose, the CO_2 is representative of the 3 and 4 carbons of the glucose. The acetaldehyde from the permanganate oxidation comes from the α and β carbons of the lactate and these carbons originated from the 1,2, and 5,6 carbons of the glucose. The aldehyde may be further split into two parts by use of the iodoform reaction which gives the 1,6 positions in one fraction and the 2,5 positions in the other. The iodoform represents carbons 1 and 6, and the formic acid, carbons 2 and 5.



A chemical degradation has likewise been worked out for application to small amounts of

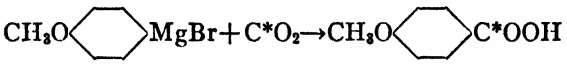
glucose (Wood *et al.*, 1945). In this method the glucose is converted to methyl glucoside and the number 3 position is split out as formic acid with cold periodic acid. Subsequent oxidation with hot periodic acid converts the primary alcohol at the 6 position to formaldehyde which may then be separated as a single fraction. The remaining carbons, 1,2,4,5 are all converted to formic acid. In application of this method the fraction representing position 3 has been found to be somewhat contaminated by carbon from other positions but nevertheless the degradation has served as a useful check on the validity of the bacterial degradation.

Results with Lower Fatty Acids and Lactic Acid

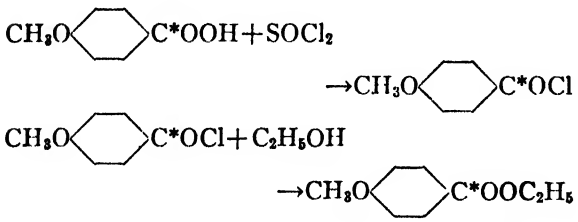
The three fatty acids used in these studies were acetate, propionate, and butyrate. Since the synthesis is a very important part of these investigations, some indication of the methods used will be given. The compounds were labeled in individual positions by syntheses which started with isotopic CO_2 and used specific reactions for locating the isotopes in the desired spot. The carboxyl-labeled acids were synthesized by the Grignard reaction

$$RMgI + C^{13}O_2 \rightarrow RC^{13}OOMgI \rightarrow RC^{13}OOH$$

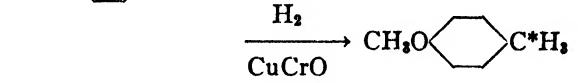
The methyl-labeled acetic acid was made by a modification of the method of Anker (1946). Anisic acid was made by the Grignard reaction



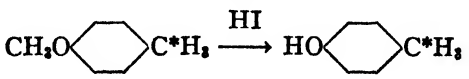
The carboxyl-labeled anisic acid was esterified by treating it first with thionyl chloride, then with ethyl alcohol.



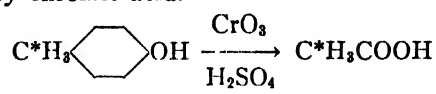
The ethyl anisate was reduced with hydrogen at 200 atmospheres pressure and 250° C, using the copper-barium-chromium oxide, 37 KAF, catalyst.



The methyl *p*-cresyl ether was hydrolyzed to *p*-cresol with hydriodic acid.



The *p*-cresol was oxidized to methyl labeled acetic acid by chromic acid.



α-labeled propionate was prepared from $CH_3C^{13}OOH$ by formation of the butyl ester and hydrogenation of the ester to the alcohols with copper-barium-chromium oxide catalyst yielding $CH_3 \cdot C^{13}H_2OH$ and $CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2OH$. The mixture of alcohols was converted to the corresponding iodides and fractionated. The ethyl iodide was converted by the Grignard reaction to propionic acid

$$CH_3 \cdot C^{13}H_2I + Mg \rightarrow CH_3 \cdot C^{13}H_2MgI + CO_2 \rightarrow CH_3 \cdot C^{13}H_2 \cdot COOH$$

β-labeled-propionate was made by the same method as the α-labeled propionate except that $C^{13}H_3 \cdot COOH$ was the starting material.

α-labeled butyrate was synthesized in a similar manner except that the starting material was $CH_3 \cdot CH_2 \cdot C^{13}OOH$.

For synthesis of β-labeled butyrate $CH_3 \cdot C^{13}OOH$ was converted to ethyl iodide as described above. The ethyl iodide was converted into butyric acid by the standard malonic ester procedure.

$$CH_3 \cdot C^{13}H_2I + Na[CH(COO \cdot C_2H_5)_2] \rightarrow NaI + CH_3 \cdot C^{13}H_2 \cdot CH(COOC_2H_5)_2$$

$$\xrightarrow{\text{hydrol.}} CH_3 \cdot C^{13}H_2 \cdot CH(COOH)_2 \xrightarrow{\text{heat}} CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot COOH + CO_2$$

Non-labeled-acetic acid from the malonic ester was separated from the labeled-butyrate by fractionation with the silica column (Elsden, 1946; Isherwood, 1946).

These labeled acids were fed to rats, and by the methods described above the glucose carbon chain was fractionated and analyzed for isotope (Lorber *et al.*, 1945; Lifson *et al.*, 1947; Wood, 1946, 1948). The results are summarized in Table 1. Briefly the procedure used was as follows. Rats were fasted for 24 hrs. and then were fed approximately 400 mg. of glucose and two to three millimoles of the fatty acid per 100 gm. of body weight. The rats were placed in a desiccator, fitted with a fan and containing alkali for collection of the respiratory CO_2 and supplied with oxygen. Samples of the respiratory CO_2 were taken each 45 min. and at the end of 2½ hrs. the livers were removed under nembutal anesthesia. The glycogen was isolated and degraded.

Only a brief summary will be given here of the results in Table 1, a more detailed discussion may

be found in the original publications and in reviews (Wood, 1946, 1948). It will be noted, in the column which diagrams the results in skeleton form, that labeled bicarbonate and all the carboxyl-labeled acids give identical patterns, *i.e.*, a sugar in which the isotope is within the limits of error exclusively in the central 3,4 positions (C—C—C*— C*—C—C). The only labeled acid other than carboxyl-labeled acids which gave this type of glucose was β-labeled butyrate. Time does not permit a full discussion of this observation but it is apparent from the pattern of isotope distribution that the β-labeled butyrate probably gives rise to a carboxyl-labeled acid by a secondary change and it is by conversion of this resultant acid that the characteristic glycogen arises. The mechanism of this conversion seems quite certainly to be by some type of β-oxidation with formation of carboxyl-labeled acetate or its derivative. There are now many lines of evidence that such a reaction occurs (Bloch and Rittenberg, 1944; Medes *et al.*, 1945; Bloch, 1947).

The other labeled acids give rise to a different glycogen; one in which the predominant concentration of isotope is in the 1,2,5,6 positions and accompanied by a lower concentration of isotope in the 3 and 4 positions (C*—C*—C′—C′—C*—C*). The concentration of isotope in the 2,5 positions apparently equals that of the 1,6 positions for each of the four labeled acids (methyl-labeled acetate, α-labeled butyrate and α and β-labeled propionate). The presence of isotope in the 3 and 4 positions could be accounted for solely by fixation of the isotopic respiratory CO_2 which arises from oxidation but it is likely that the isotope enters through other mechanisms as well. This suggestion is supported by the fact that the isotope content of the respiratory CO_2 bears no constant relationship to that of the 3 and 4 positions. The mechanism of the introduction of isotope in the sugars will be considered later.

It is interesting to note that β-oxidation of α-labeled butyrate would give rise to a methyl-labeled acetate and that in this case a glycogen similar to that of methyl-labeled acetate should arise from the butyrate. The fact that this does occur lends further weight to the idea that butyrate is metabolized in the intact animal largely by β oxidation.

$$CH_3 \cdot CH_2 \cdot C^{13}H_2 \cdot COOH \rightarrow CH_3 \cdot COOH + C^{13}H_3 \cdot COOH \rightarrow C^*—C^*—C'—C'—C^*—C^*$$

It is thus seen that from the determination of the isotope distribution of glycogen some ideas on the mechanism of metabolism of butyrate may be derived.

TABLE 1. DISTRIBUTION OF C^{13} IN GLUCOSE UNIT OF RAT LIVER GLYCOGEN

C^{13}-compound fed	Percent C^{13}	Percent of C^{13} in glucose (from glycogen)			Location of isotope in the glucose
		3,4	2,5	1,6	
$NaHC^*O_3$	5.10	.16	.00	−.01	C C C* C* C C
CH_3C^*OOH	2.63	.14	.01†	.01†	C C C* C* C C
C^*H_3COOH	2 02	.08	.18	.16	C* C* C′ C′ C* C*
$CH_3CH_2C^*OOH$	1.54	.15	−.01†	−.01†	C C C* C* C C
$CH_3C^*H_2COOH$	1.56	.07	.26	.27‡	C* C* C′ C′ C* C*
$C^*H_3CH_2COOH$	0.74	.04	.17	.15	C* C* C′ C′ C* C*
$CH_3CH_2CH_2C^*OOH$	0.98	.13	.01	.01	C C C* C* C C
$CH_3CH_2C^*H_2COOH$	0.74	.05	.16	.14	C* C* C′ C′ C* C*
$CH_3C^*H_2CH_2COOH$	1.09	.16	.02	.02	C C C* C* C C

† Combined carbons of 2,5 and 1,6 were analyzed, the results being the average of the four positions.
‡ Value for carbon 6 by chemical degradation. The CHI_3 from the bacterial degradation was contaminated by accident.

Before consideration is given to the results with propionate, the results with different types of labeled lactate will be reviewed. It is important to consider these results in conjunction with those of propionate because one possible mechanism for converting propionate to glycogen is by α oxidation to pyruvate or lactate with a subsequent conversion to glycogen.

The α-labeled lactate was prepared from $CH_3 \cdot C^{13}H_2 \cdot COOH$ which was synthesized as previously described. The sodium propionate was brominated and the bromine was replaced by a hydroxyl.

$$CH_3 \cdot C^{13}H_2 \cdot COONa \xrightarrow{P \text{ and } Br} CH_3 \cdot C^{13}HBr \cdot COOH \xrightarrow{Na_2CO_3} CH_3 \cdot C^{13}HOH \cdot COONa$$

The α,β-labeled lactate was made by a modification of the method of Cramer and Kistiakowsky (1941).

$$BaC^{13}O_3 \xrightarrow[\text{hydrol.}]{\text{heat Mg}} BaC_2^{13} \longrightarrow HC^{13}\equiv C^{13}H \xrightarrow[H_2O]{HgSO_4} C^{13}H_3C^{13}HO \xrightarrow{HCN} C^{13}H_3\cdot C^{13}HOH\cdot CN \xrightarrow[HCl]{HOH} C^{13}H_3\cdot C^{13}HOH\cdot COOH$$

The results given in Table 2 show that from α-labeled lactate a sugar is formed in which the 2,5 positions carried the predominant concentration of isotope, the 1,6 positions less and the 3,4 positions the least (C^0—C^*—C'—C'—C^*—C^0). In the case of the α,β-labeled lactate the labeling is equal in 1,6 and 2,5 with a lower concentration in 3,4.

Discussion of Mechanisms

Before considering the explanation of the results with lactate the results with the other acids will be reviewed briefly in terms of the scheme shown in Diagram 1, which is an abridged version of the relationships of the tricarboxylic acid cycle and glycogen synthesis. According to this scheme butyrate and acetate enter glycogen via the tricarboxylic acid cycle through reactions *k, f, g, h, i, e, d, c, b, a*. Time will not be taken to discuss this mechanism in detail since this has been done elsewhere (Wood, 1946, 1948; Buchanan and Hastings, 1946), but study of the scheme will show that carboxyl-labeled acetic acid or acids such as butyric acid, which following β oxidation yield carboxyl-labeled acetic acid, will give rise to carboxyl-labeled oxalacetate, which in turn gives carboxyl-labeled pyruvate by reaction *b*. The pyruvate is converted to glucose by reaction *a*. Reaction *a* represents the many steps of the overall conversion of glycolysis in which the net result is the joining of two pyruvate molecules carboxyl to carboxyl; thus giving from carboxyl-labeled pyruvate a 3,4-labeled sugar.

Through the same series of reactions with methyl-labeled acetate or a butyrate which yields methyl-labeled acetate, an oxalacetate is formed in which the two central carbons are labeled. This compound can then go directly to pyruvate by reaction *b* or it may re-enter the cycle by reaction *f*. Study of the scheme will show that one of the central carbons, as a result of recycling, becomes a carboxyl group of oxalacetate. In this way methyl-labeled acetate gives rise to an oxalacetate which is labeled on the average in all positions, the heaviest concentration being in the central position since the initial introduction of isotope is in this position. In the central two carbons of oxalacetate the concentration of isotope is equal since the oxalacetate is derived from succinate which is symmetrical. Thus the pyruvate that is formed by reaction *b* is equally labeled in the α and β positions and by reaction *a*, gives rise to a glucose which is labeled equally in the 1,6 and 2,5 positions. The 3,4 positions contain a lower concentration of isotope.

For brevity this above explanation is presented in a rather dogmatic manner and the author wishes to emphasize that it is recognized that this is but a tentative scheme and probably is incomplete in many respects. Nevertheless it explains quite well the results so far obtained experimentally.

Table 2. Distribution of C^{13} in the Glucose Unit of Rat Liver Glycogen

C^{13}-compound fed	Percent C^{13}	Percent of C^{13} in glucose (from glycogen)			Location of isotope in the glucose
		3,4	2,5	1,6	
$CH_3\cdot C^*HOH\cdot COOH$	1.55	.05	.27	.18	C^0 C^* C' C' C^* C^0
$C^*H_3\cdot C^*HOH\cdot COOH$	3.37	.15	.60	.59	C^* C^* C' C' C^* C^*

The explanation of the lactate results may next be considered. Since lactate and pyruvate are probably in equilibrium with each other the lactate will be discussed in terms of pyruvate conversions. Pyruvate may undergo three conversions in the scheme, *i.e.*, oxidative decarboxylation to an acetyl group by reaction *i*, conversion to oxalacetate by the fixation reaction *b*, or direct conversion to glucose by reaction *a*. These three reactions lead to formation of three different types of sugar.

The direct conversion of $CH_3\cdot C^{13}O\cdot COOH$ to glucose by reversal of glycolysis, reaction *a*, would yield the following sugar C—C^*—C—C—C^*—C, the carbonyl group of pyruvate occupying the 2 and 5 positions.

Reaction *j* ($CH_3\cdot C^{13}O\cdot COOH \rightarrow CH_3\cdot C^{13}OX + CO_2$) gives a carboxyl-labeled acetyl group which by the mechanisms of the cycle would give a carboxyl-labeled oxalacetate and a C—C—C^*—C^*—C—C labeled sugar, just as does $CH_3\cdot C^{13}OOH$.

Reaction *b* would give oxalacetate with a label in the carbonyl carbon. It may then undergo two transformations (1) via *f* through the complete reactions of the cycle, (2) the reversible reactions *c, d, e*, to succinate where the irreversible reaction *i* stops further transformation in that direction. Both these series of reactions lead to formation

of the same type of oxalacetate, *i.e.*, one with the central carbons equally labeled. Thus in the round trip to fumarate and back through reversible reactions *c* to *d*, two types of oxalacetate ($COOH \cdot C^{13}H_2 \cdot CO \cdot COOH$ and $COOH \cdot CH_2C^{13}O \cdot COOH$) would be formed and the isotope on the average would be distributed equally in both central carbons of the oxalacetate. Likewise as the scheme is written, the same thing happens to the carbonyl-labeled oxalacetate which goes around the cycle via reaction *f*, etc. and the isotope is equally distributed in the central carbons. From this type of oxalacetate $C^*H_3 \cdot C^*O \cdot COOH$ would be formed in which the isotope concentration would be equal in the α and β positions and this pyruvate would give a sugar labeled as follows: C*—C*—C—C—C*—C*.

The resultant glycogen of the above described conversions therefore would be the average of three types of sugars:

1. C—C*—C—C—C*—C
2. C—C—C*—C*—C—C
3. C*—C*—C—C—C*—C*

The highest concentration would be expected in positions 2 and 5 since the primary source of isotope is α-labeled lactate which would give a sugar of type 1.

With the α-, β-labeled lactate the mixture would consist of only types 2 and 3 and the concentration would be expected to be equal in 1,6 and 2,5; a result which was realized.

It is interesting that the isotope distribution in the glucose gives some indication of how much of the administered isotope goes into the sugar by the respective routes. If one considers for the moment only types 1 and 3 and the isotope concentration for position 1,6 and 2,5 of the example given in Table 2, it is seen that the concentration is higher in 2,5 by a value of .09 than it is in the 1,6 positions (.27 compared to .18). If this extra C^{13} of the 2,5 positions (.09) had been distributed equally in the 1,6 and 2,5 positions the extra concentration would have been .045. When .045 is compared to .18 it is seen that one part of the isotope found in the glycogen in carbons, 1,2,5,6, got in by the direct route as compared to 4 parts by the indirect routes which involved symmetrical molecules. These results are in good agreement with those of Boxer and Stetten (1944), who found by measuring the rate of incorporation of deuterium from body water that glycogenesis occurs from small fragments in the fasted animal. Viewed in the sense of the Boxer and Stetten experiments if the pyruvate went around the cycle or entered into reactions *b* to *d* many of the hydrogens would be labilized and exchange would occur with deuterium of the body water. Thus a considerable quantity of deuterium would be incorporated in the glycogen and this was their indicator of the amount of synthesis that was occurring from small fragments.

The present results indicate the relative proportion of the administered isotope that goes to glycogen by the different routes, but they do not necessarily give a true indication of the total glycogen that comes from each route. For example, it does not seem improbable that the lactate which enters the glucose and glycogen by the direct route may mix with less unlabeled compounds and thus undergoes less dilution of the isotope than would be the case by the indirect route. If such were the situation a given amount of isotope would represent a smaller quantity of actual material on the part of the direct route than the same amount of isotope would for the indirect route. Actually to compare the amount of material transferred by each route it would be necessary to know the relative dilution of isotope by the two routes, and the present experiments give no indication of these respective dilutions.

Another example of how the distribution of isotope in glucose may be used to give information about the mechanism of carbohydrate metabolism is illustrated by the following case.

It was formerly believed that phosphopyruvate could not be formed directly from pyruvic acid through phosphorylation with ATP (Meyerhof *et al.*, 1938). As a consequence a mechanism of formation of phosphopyruvate was proposed (Solomon *et al.*, 1941) whereby the phosphopyruvate was formed by the following reactions which involved conversion of the pyruvate to oxalacetate by CO_2 fixation, conversion of the oxalacetate to fumarate and finally oxidative phosphorylation of the fumarate to give phosphopyruvate.

$$CO_2 + CH_3 \cdot C^{13}O \cdot COOH \rightleftharpoons COOH \cdot CH_2 \cdot C^{13}O \cdot COOH$$

$$COOH \cdot CH_2 \cdot C^{13}O \cdot COOH \rightleftharpoons COOH \cdot CH{:}C^{13}H \cdot COOH$$

$$COOH \cdot CH{:}C^{13}H \cdot COOH \xrightarrow[-2H]{H_3PO_4} \begin{matrix} CH_2{:}C^{13}(OPO_3H_2) \cdot COOH + CO_2 \\ \text{and} \\ C^{13}H_2{:}C(OPO_3H_2) \cdot COOH + CO_2 \end{matrix}$$

It can be seen from the above reactions that because of the symmetrical nature of fumarate this series of steps would give a phosphopyruvate which is on the average labeled equally in the α and β positions. Thus, if these reactions were an obligatory mechanism for formation of phosphopyruvate and the only route for synthesis of glucose were via phosphopyruvate, the concentration of isotope would of necessity be equal in the 1,6 and 2,5 positions. Since this distribution of isotope was not found for α-labeled lactate, it seems reasonably certain that phosphopyruvate does not arise exclusively via fumarate. This deduction assumes of course that all glycogen synthesis occurs via phosphopyruvate.

Actually Lardy and Ziegler (1945) have demonstrated that the direct phosphorylation of pyruvate with ATP does occur. The present results with intact animals are in agreement with the results of Lardy and Ziegler in that they show that pyruvate may be used directly for glycogen synthesis in the inact animal.

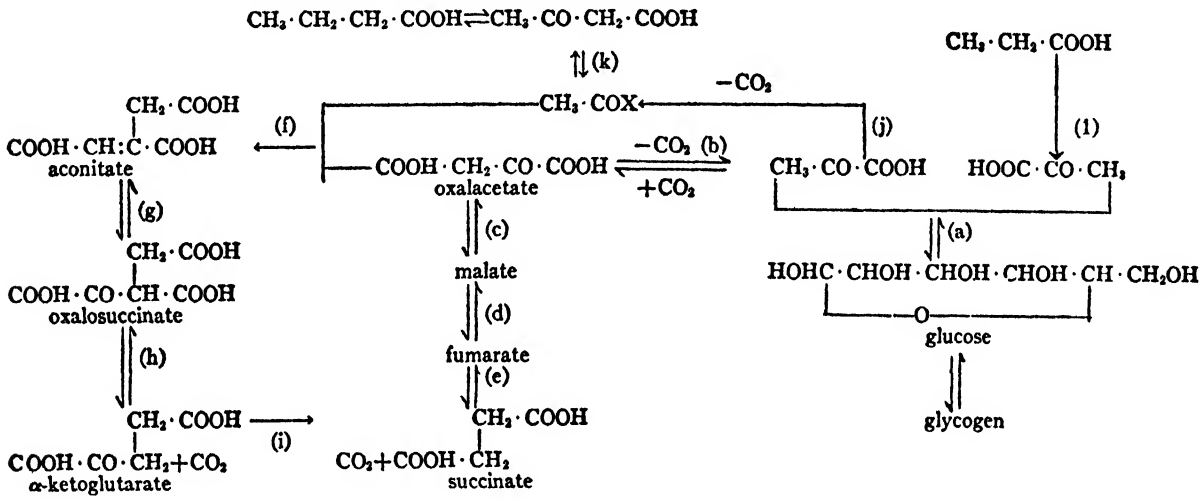
With the results from lactate in mind it is possible to more clearly consider the data from the propionate experiments. Propionate is definitely glycogenic but the mechanism of conversion is unknown. It is indicated in Diagram 1, for lack of

carboxyl- and β-labeled propionate should in that case give the same labeled malonic acid and thus the same labeled glycogen. This was not realized (Table 2).

$$C^{13}H_3\cdot CH_2\cdot COOH \searrow$$
$$COOH\cdot CH_2\cdot C^{13}OOH$$
$$CH_3\cdot CH_2\cdot C^{13}OOH \nearrow$$

The equal distribution of isotope in the 1,6 and 2,5 positions indicates the possibility that pro-

Diagram 1. Abridged Tricarboxylic Acid Cycle and its Relation to Glycogen Synthesis



better suggestion, that propionate is oxidized to pyruvate. Bloch and Rittenberg (1944) on the basis of deuterium experiments have concluded that propionate is not converted to pyruvate by α oxidation. Actually the present results might also be considered inconsistent with the indicated mechanism. Whereas with α-labeled lactate, a glycogen was obtained which contained the highest concentration of isotope in the 2,5 positions, with α-labeled propionate and β-labeled propionate the distribution was equal in the 1,6 and 2,5 positions. This fact raises the question of whether pyruvate is a direct product of propionate metabolism because if it were, one might anticipate a similar type of labeled-glycogen would be formed from propionate and pyruvate. This is not necessarily the case however, because the rates of reaction may be such that pyruvate as formed from propionate may come to equilibrium with a symmetrical molecule and thus randomize the isotope. Whereas when lactate is given, the concentration of pyruvate may be higher and force some of the pyruvate directly into the glycolysis reactions.

The results do exclude the β oxidation of propionate to malonic acid and subsequent conversion of the malonic acid to glycogen because

pionate conversion to glycogen may entail a symmetrical dicarboxylic acid as part of the mechanism. It is interesting that Johns (1948) has recently found a microorganism that very actively decarboxylates succinate to propionate. A similar but slow reaction was described earlier for the propionic acid bacteria by Werkman and Wood (1942), *cf.* p. 151. If such a reaction were reversible it would provide a possible mechanism for converting propionate to glycogen and at the same time would account for the equal distribution of the isotope in positions 1,6 and 2,5

$$CO_2+CH_3\cdot C^{13}H_2\cdot COOH$$
$$\rightleftarrows COOH\cdot C^{13}H_2\cdot CH_2\cdot COOH$$
$$\rightleftarrows COOH\cdot C^{13}H{:}CH\cdot COOH$$
$$COOH\cdot C^{13}HOH\cdot CH_2\cdot COOH$$
$$\rightleftarrows \quad \text{and}$$
$$COOH\cdot CH_2\cdot C^{13}HOH\cdot COOH$$
$$\rightleftarrows COOH\cdot C^{13}H_2\cdot C^{13}O\cdot COOH$$
$$\text{average}$$
$$\rightleftarrows C^{13}H_3\cdot C^{13}O\cdot COOH$$
$$\text{average}$$
$$\rightleftarrows C^*—C^*—C—C—C^*—C^*$$
$$\text{average}$$

There is no evidence for the first reaction at the present time. It is clear from the above discussion that our knowledge of the mechanism of propionate conversion to glycogen is very limited and additional work is needed on this problem.

Conversion of Glycine to Glycogen

Before concluding this discussion of the synthesis of glycogen, I wish to present some results obtained recently by Dr. Sakami and Dr. Lorber, relative to glycine metabolism. Glycine is an interesting compound because it is the only two carbon compound which is agreed to be glycogenic by most investigators. In contrast there is no agreement that acetate is glycogenic, *i.e.*, in the sense that it will induce a net increase in glycogen following its administration. As pointed out by Buchanan *et al.* (1945), the scheme in Diagram 1 does not provide for a net increase since the fed compound is transformed and mixed with intermediates of the metabolic pool. Viewed in this light there is no reason to expect a direct correspondence between the amount of isotope and the total net increase in a body constituent, because the identical carbon fed is not necessarily, and probably will not be, the carbon incorporated into the compound which is observed to increase.

With labeled-glycine it seemed possible that some indication of the mechanism of glycine metabolism might be gained by determining the isotope distribution in the glycogen. Table 3 shows results from fasted rats which were given approximately six mM of carboxyl-labeled glycine per 100 gm. weight and were sacrificed 12 hrs. later for analysis of their liver glycogen. The glycine was synthesized from carboxyl-labeled acetate by the method of Sakami *et al.* (1947). It is seen from the data there there is nothing distinc-

Table 3. Distribution of C^{13} in the Glucose Unit of Rat Liver Glycogen

C^{13}-compound fed	Percent C^{13}	Percent of C^{13} in glucose (from glycogen)			Location of isotope in the glucose
		3,4	2,5	1,6	
$CH_2NH_2 \cdot C^{13}OOH$	1.95	.41	.02	.00	C C C* C* C C

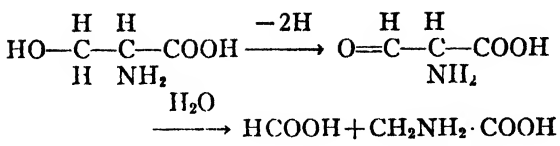
two molecules of CO_2 are lost in the transformation of acetate to glycogen via the cycle. Thus the net transfer of carbon to glycogen is zero. The fact that isotope from acetate is converted to glycogen might seem inconsistent, but the reason the isotope is transferred is because the carbons lost as CO_2 in the cycle are not identical with those of the acetate. Thus there is isotope transfer in spite of no net transfer of carbon.

Glycine is of additional interest because it exhibits a delayed reaction in glycogen formation. The peak of the glycogen deposition has been shown by MacKay *et al.* (1940) to be 14 hrs. after the feeding. Olsen *et al.* (1943) have fed carboxyl-labeled glycine to mice and found the glycogen to contain excess isotope. The recovery of the fed isotope in the glycogen amounted to about one percent and because of this low recovery it was concluded that the ingested glycine in some way promoted the formation of glycogen from other body constituents. This might be considered a possible explanation of the delayed glycogen formation. Actually the same low recovery is found when either carboxyl-labeled lactate (Conant *et al.*, 1941) is fed or carboxyl-labeled alanine (Gurin *et al.*, 1947) and these compounds do not give a delayed glycogen or sugar formation. Thus the low isotope recovery is not peculiar to glycine and it may have no bearing on the delayed reaction. The low recovery of isotope is usually assumed to be due to the dilution of isotope which occurs when

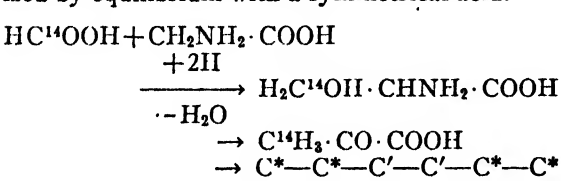
tive about the isotope distribution pattern. The isotope is in positions 3,4 just as it was for the other carboxyl-labeled acids.

The fact that the isotope appeared only in positions 3,4, and that glycine is glycogenic indicated that the conversion probably occurred by some reaction which did not involve the CO_2 loss of the cycle. Since Shemin (1946) had shown with serine labeled in the carboxyl and nitrogen groups, that glycine is formed from serine by loss of the β carbon, it seemed possible that the serine-glycine reaction might be reversible and involve the addition of a 1-carbon compound. It is known that serine can be deaminated anaerobically (Binkley, 1943; Chargaff and Sprinson, 1943) to yield pyruvic acid. The conversions of glycine to serine to pyruvate to glycogen appeared to be a possible mechanism for formation of glycogen from glycine. These reactions would give a net transfer of glycine to glycogen and introduce the carboxyl group into the 3,4 positions of the glucose. The 1-carbon compound obviously is not CO_2 because the amount of isotope introduced into the 1,6 and 2,5 positions is extremely small; with radioactive carbon dioxide it has been found that the 3,4 carbon contains more than 96 percent of the fixed CO_2 of the glycogen (Shreeve *et al.*, 1948).

One of the reactions suggested by Shemin (1946) for the formation of glycine was oxidation followed by hydrolytic cleavage to formic acid and glycine.



It was decided to test formic acid and glycine in combination for incorporation of formic acid carbon in glycogen. It would be expected if the mechanism of glycine conversion was by the reverse of the above reaction with subsequent conversion to pyruvate and glycogen that the isotope of formic acid would appear in the 1,6 and 2,5 positions with the highest concentration in the 1,6 positions providing the isotope was not randomized by equilibrium with a symmetrical acid.



2.5 mM of glycine was fed and 0.25 mM of C^{14} formic acid containing 80,100 counts per mg. C per minute was given intraperitoneally, *i.e.*, a total of 240,000 counts per 100 gm. The respiratory CO_2 was collected and the glycogen was isolated after 14 hrs. The respiratory CO_2 in the first hour contained 1,000 counts per mg. C per minute, by the third hour it had dropped to 376 counts, by the fifth it was 95, the eighth 51, and the eleventh 26. The glycogen degradation figures were as follows: 3,4=256 counts per mg. C per minute, 1,2,5,6=336 counts per mg. C per minute. The fact that the activity was relatively low in relation to the initial activity of the formate can not be evaluated in terms of the quantitative significance of the reaction until more is known about the dilution of the C^{14} by metabolic precursors of the glycogen and whether formic acid as such is the biological form of the 1-carbon compound. If formic acid were not the normal biologically active compound the amount of C^{14} incorporated would depend upon the rate of conversion of the C^{14} formate to the normal intermediate.

At any rate the finding of isotope in positions 1,2,5,6 is extremely interesting and the results are compatible with a mechanism in which formic acid or a derivative reacts with glycine. It is clear at least that the carbon of formic acid can be incorporated in glucose and it is quite certain that the carbon was at one time in the α or β or both positions of pyruvic acid.

The presence of C^{14} in the respiratory CO_2 indicates that formic acid is oxidized to CO_2. This CO_2 might be the source of the isotope in the 3,4 positions.

Since the experimental conditions were different than those of the previous experiments it remained possible that under the conditions of the glycine experiments CO_2 was fixed in positions besides 3,4. In order to have results more comparable to those of the other experiments and as a check on this possibility, rats were fed glucose, and formic acid was injected intraperitoneally. The glycogen was isolated after three hours. In this experiment the formate was less active than in the previous experiment and approximately .5 as much total activity was given. The conditions were as follows: .5 gm. of glucose was fed per 100 gm. of rat and 1.45 mM of formate with 6,220 counts per mg. C per minute was given, *i.e.*, a total of 108,500 counts per 100 gm. The following results were obtained. The respiratory CO_2 contained 194 counts per mg. C per minute in the first hour, 271 the second and 256 the third. The 3,4 positions of the glucose contained 55 counts per mg. C per minute, the 1,2,5,6 carbons 33 counts. In addition the 1,6 and 2,5 fractions were obtained and preliminary results indicate that the activity was about equal in the two fractions. This would seem to indicate, if serine is formed with formate carbon in the β position, that the isotope becomes randomized during its conversion to glycogen. The important point is that activity occurred in the 1,2,5,6 positions under conditions in which little or no CO_2 carbon enters these positions. This fact indicates that the formate carbon does not get into the 1,2,5,6 positions via CO_2. It thus appears well established that formate carbon enters the 1,2,5,6, positions by some mechanism other than CO_2 fixation.

The ratio between the activity of the respiratory CO_2 and that of the 3,4 positions was quite similar to that obtained in isotopic bicarbonate experiments and from this viewpoint it seems reasonable to assume that most of the isotope entered the 3,4 positions by fixation of CO_2.

Returning now to a consideration of the glycine experiment, there is one problem with respect to it for which the explanation is at present obscure. This problem relates to the delayed deposition of glycogen following glycine feeding. MacKay *et al.* (1940) found that there is practically no deposition of glycogen until after the sixth hour and the maximum is not reached until the fourteenth hour. It is to be noted in the glycine experiment that the counts were 256 per mg. C per minute in the 3,4 positions whereas in the respiratory CO_2 at the fifth hour the counts were only 95 per mg. C per minute. At the eighth hour they were 51. Since the greatest glycogen deposition would be expected at about the eighth hour, glucose was apparently being formed which contained more isotope in the 3,4 positions than did the respiratory CO_2. This raises a doubt as to whether or not the C^{14} of the center positions actually came from CO_2 in the glycine experiments. If it did come from the CO_2 it would seem likely that the fixation must have occurred in a glycogen precursor at an early time and retained the activity during the subse-

quent conversion to glycogen. The mechanism whereby isotope may be transferred to glycogen without coming to equilibrium with the general metabolic pool (as judged by the isotope concentration of the respiratory CO_2) is extremely interesting just as is the whole question of delayed glycogen deposition. It is probable that the isotope concentration of the respiratory CO_2 may not be representative of liver metabolism *per se* since the respiratory CO_2 arises from the combined metabolism of the whole body. Synthesis from the administered isotopic compounds probably occurs first in the liver and the isotope concentration in the organic compounds may therefore be highest in this organ. Thus at the eighth hour the isotope concentration may have been greater in the liver than in the other organs and also the metabolic CO_2 of the liver may have been higher than that of the general body metabolism. From these considerations it is obvious that further work is needed both on the problem of the delayed glycogen storage and its relation to isotope concentrations.

In addition to the indirect suggestion from the glycogen experiment that formic acid is incorporated in serine, somewhat more direct evidence has been obtained by periodic acid oxidation of the hydrolyzed liver protein. Formaldehyde obtained by this reaction has been found to contain C^{14}. Although serine is the only known amino acid which contains a primary alcohol, hydroxy proline also yields formaldehyde on periodic acid oxidation (Carter and Neville, 1947), as does glucose amine which might contaminate the amino acids. Although these results are not conclusive, they indicate that C^{14} probably is fixed in the β position of the serine.

An additional experiment has confirmed rather conclusively the presence of fixed formate in the serine. In this experiment four rats were used which were given per 100 g. of rat .25 mM of C^{14} formate containing a total of 407,000 counts and 5 mM of N^{15} and C^{13} labeled glycine ($CH_2 \cdot N^{15}H_2 \cdot C^{13}OOH$). The glycogen from the liver was isolated and C^{14} was found present in it as in the other experiments. In addition the amino acids of the proteins of the liver were worked up.

The serine has been isolated as the parahydroxyazobenzenesulfonate. Its decomposition temperature when determined simultaneously with a derivative from known L-serine was found identical with that of the known derivative at 204°. This derivative contained approximately 134 cts/mg. serine carbon/minute; the self absorption of the derivative is not known and the self absorption was assumed to be the same as $BaCO_3$ in this calculation. These results seem to conclusively show that formate is converted to serine; the only likely source of error is co-precipitation of a highly active material.

The degradation of the serine has not been completed as yet but since the degradation procedure will be fairly specific for serine the possibility of error will be further eliminated at this step. After the degradation is complete it will be possible to compare the rate of entrance of formate and glycine. By this means it is hoped adequately to establish whether or not the formation of serine occurs by union of formic acid carbon and glycine.

It should be noted that Greenberg and Winnick (1948) have already reported that C^{14} glycine is not converted to serine in rats. If these results are correct, then the entrance of formate in glycogen by combination with glycine is not an acceptable explanation of our results.[1] On the other hand Ehrensvärd *et al.* (1947) have found after addition of carboxyl-labeled glycine to Torulopsis yeast and incubation for three hours that of five amino acids isolated from the yeast, serine and proline contained by far the highest concentration of C^{13}. They suggest that glycine may be converted to serine and to proline. Since proline is thought to give rise to α-ketoglutarate (Taggart and Krakaur, 1947) and α-ketoglutarate is converted to glycogen, this might provide a mechanism for glycine to get into glycogen. However this mechanism would not offer, on the basis of present information, an explanation for the formic acid fixation in the glycogen.

The present results together with those of others indicate that formate may play an important role in metabolism. Buchanan and Sonne (1946) have shown the incorporation of formate in purines and Gordon *et al.* (1948) have revealed a possible function of folic acid as a formyl carrier in the purine synthesis.

Summary

Study of the distribution of carbon isotopes in glycogen following feeding of labeled compounds has proved to be a useful method for investigation of metabolism of labeled compounds in the intact animal. It has been possible by this method to provide considerable evidence for the occurrence *in vivo* of mechanisms which were derived largely by *in vitro* methods. The fixation of formate in glycogen has been uncovered by this mechanism and the distribution of the isotope is such as to indicate the mechanism may be by conversion of formate and glycine to serine and the latter to glycogen. Using C^{14} formate the conversion of formic acid to serine has been demonstrated.

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[1] Winnick, T., Moring-Claesson, I., and Greenberg, D. J. (1948, Distribution of radioactive carbon among certain amino acids of liver homogenate protein, following uptake experiments with labeled glycine, J. biol. Chem. *175:* 127-132) have shown the conversion of glycine to serine using liver homogenates. In addition, Sakami, W. (1948, Conversion of formate and glycine to serine and glycogen in the intact rat, J. biol. Chem. *176:* 995-996) has presented definite evidence that both formate and glycine are fixed in serine *in vivo*.

Extension of this method to study of conversion of other compounds to glycogen has important possibilities. A similar study on blood sugar offers promise for study of metabolism in humans and in clinical medicine.

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